Universidade de Aveiro Departamento de Biologia
2013

Mariana Sofia Oliveira Pandeirada

Studies on freshwater woloszynskioids (Dinophyceae)

Estudo de woloszynskióides de água doce (Dinophyceae)

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Aveiro, 27 de Novembro de 2013

(Mariana Sofia Oliveira Pandeirada)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Ecologia, Biodiversidade e Gestão de Ecossistemas, realizada sob a orientação científica do Professor Doutor António José de Brito Fonseca Mendes Calado, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro

Aos meus avós, pais e irmã

The Biology of Algae

The biology of algae is a duty, or a task,
That consumes the better portion of your time
In the sampling of waters from an ocean, or a flask,
Or a snow-field, or a gutter-full of slime.
You get cold, and wet, and grubby; you get dusty, hot, and dry;
You get dismal, and dejected, and defied;
But you'll find that, if you're lucky – if you're good – and if you try,
You can do a little science on the side.

The biology of algae is a pastime, or an art,
That embodies a diversity of skill:
How to mend a pH meter which has somehow come apart,
Or to regulate a microscope or still;
How to edit a proposal, or a chapter of a book;
How to float upon the academic tide;
How to teach a fellow creature how to speak, or how to cook,
And a little bit of science on the side.

The biology of algae is a virtue, or a vice,
That entails some tricky searching of the soul.
It involves the growth of fishes, and the harvesting of rice,
And pollution, and the origins of coal.
It may get us into trouble; it may get us into space;
Its dilemmas are as long as they are wide.
It involves some moral judgements on the future of our race –
And a little bit of science on the side.

Ralph Lewin (R.A.L. 1971. *Phycol. Newsletter* 7:1)

o júri/ the jury

presidente/ president

Prof. Doutor João António de Almeida Serôdio
Professor auxiliar do departamento de Biologia da Universidade de Aveiro

vogais/ members

Prof. Doutor Jorge Manuel Estima de Almeida Rino
Professor associado aposentado do departamento de Biologia da Universidade de Aveiro

Prof. Doutor António José de Brito Fonseca Mendes Calado (Orientador)
Professor auxiliar do departamento de Biologia da Universidade de Aveiro

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palavras-chave

Borghiella andersenii, ciclo de vida, dinoflagelados, filogenia, morfologia, taxonomia, *Tovellia aveirensis*, ultraestrutura, woloszynskióides.

resumo

Os dinoflagelados são um grupo de protistas com características intra- e extracelulares invulgares, encontrados tanto em habitats marinhos como de água doce. Estes organismos são tradicionalmente classificados como tecados ou atecados tendo em conta a constituição da sua região externa, conhecida como anfiesma. Esta região compreende uma camada única de vesículas achatadas subjacentes ao plasmalema, as quais podem ser preenchidas com placas celulósicas mais ou menos espessas nos dinoflagelados tecados, ou com placas celulósicas muito finas, ou mesmo não possuírem placas, nos dinoflagelados atecados. Durante o século XX, contudo, foi demonstrado que algumas espécies atecadas do género *Gymnodinium* tinham um anfiesma constituído por numerosas placas celulósicas finas. Tais espécies foram transferidas para um novo género, *Woloszynskia*, o qual foi posteriormente objeto de controvérsia, principalmente associada com o estabelecimento da espécie tipo e a possibilidade de compreender outros grupos taxonómicos, sendo portanto polifilético. Recentemente, uma série de estudos confirmaram a última ideia, e vários géneros foram criados para receber espécies de *Woloszynskia*, conhecidas como woloszynskióides. Esses géneros foram distribuídos por diferentes famílias: *Tovellia*, *Jadwigia, Esoptrodinium* e *Opisthoaulax* na nova família Tovelliaceae; *Borghiella* e *Baldinia* na nova família Borghiellaceae; *Biecheleria* e *Biecheleriopsis* incluídos na família Suessiaceae. Estas mudanças taxonómicas foram suportadas por dados moleculares e diferenças morfológicas na estrutura do estigma, organização do apex da célula e tipo de quisto de resistência.

O conhecimento taxonómico sobre a diversidade e distribuição de dinoflagelados de água doce em Portugal Continental foi reunido pela primeira vez numa "checklist" e aqui apresentado (Capítulo 2). As entradas na lista foram definidas tendo em conta pesquisa filogenética recente, em particular mudanças taxonómicas que afetam os limites a nível genérico dos taxa. Registos publicados de espécies de dinoflagelados de água doce, retirados de 37 referências, formam a base do inventário, aos quais foi adicionada documentação para 12 taxa ainda não referenciados para Portugal (11 espécies e uma forma).

Duas novas espécies de woloszynskióides para a ciência, não incluídas nesta "checklist", são aqui apresentadas (Capítulos 3, 4). A morfologia das células e quistos é descrita, bem como a ultraestrutura das células móveis e aspetos particulares do ciclo de vida. Filogenias baseadas em sequências de LSU rDNA confirmam as novas espécies.

resumo (continuação)

A primeira espécie pertence à família Tovelliaceae, género *Tovellia* (Capítulo 3). O epíteto específico escolhido foi *aveirensis*, o qual constitui uma referência ao nome da universidade, bem como da cidade onde foi encontrada: Campus da Universidade de Aveiro, Aveiro, Portugal. *Tovellia aveirensis* possui a característica peculiar de produzir um quisto de resistência com paracíngulo e ornamentado com numerosos processos ramificados, que não só difere do quisto bipolar e quase não ornamentado do género, mas também de todos os outros descritos para woloszynskióides. Morfologicamente esta difere de outras espécies de *Tovellia* principalmente por ter uma linha de pontos posicionada ao nível do limite posterior do cíngulo, rodeando a célula, e por não possuir uma placa antapical distinta, à volta da qual as séries de placas do hipocone poderiam estar dispostas.

A segunda espécie de woloszynskióide foi encontrada na área alagada do Ribeiro da Palha, Nariz, Aveiro, Portugal, e num lago de água doce na Escócia (Capítulo 4). Esta pertence à família Borghiellaceae, género *Borghiella*, e foi nomeada *B. andersenii* em honra do Prof. Robert A. Andersen, que primeiro estabeleceu cultura da mesma a partir de material colhido na Escócia. Morfologicamente é idêntica à *B. dodgei*, divergindo desta principalmente por ter um epicone arredondado e um par de vesículas anfiesmais alongadas (PEV) mais curto, com menos pontos e delineado por duas a três placas apicais. *B. andersenii* é capaz de se reproduzir assexuadamente tanto no estado móvel, por fissão, como no estado imóvel, com produção de quistos de divisão, algo que nunca foi referenciado para Borghiellaceae. Além disso, evidências mais fortes de reprodução sexuada para esta família foram ainda observadas em culturas de *B. andersenii*, nomeadamente planozigotos e aparentes quistos de resistência.

Dois outros woloszynskióides, designados MSP1 e MSP12, são aqui brevemente descritos (Capítulo 5). Estes foram colhidos respetivamente num lago da Gafanha da Boavista, próxima da Vista Alegre, Ílhavo, Aveiro, e no mesmo local, em Portugal, onde *B. andersenii* foi encontrada. Tanto os resultados morfológicos como filogenéticos sugerem que são duas novas espécies de *Tovellia*, evolucionariamente próximas de *T. aveirensis*.

keywords

Borghiella andersenii, dinoflagellates, life cycle, morphology, phylogeny, taxonomy, *Tovellia aveirensis*, ultrastructure, woloszynskioids.

abstract

Dinoflagellates are a group of protists with intra- and extracellular unusual features, found in both marine and freshwater habitats. These organisms are traditionally classified as armoured or thecate, and unarmoured or athecate taking into account the constitution of their outer region, known as amphiesma. This region comprises a single layer of flat vesicles underlying the plasmalemma, which can be filled with more or less thick cellulosic plates in the thecate dinoflagellates, or with very thin cellulosic plates or no plates at all in the athecate ones. During the 20th century, however, it was demonstrated that some athecate species of the genus *Gymnodinium* had an amphiesma constituted by numerous thin cellulosic plates. Such species were transferred to a new genus, *Woloszynskia*, which has been later object of controversy, mainly associated with the establishment of the type species and the possibility to comprise other taxonomic groups, thus being polyphyletic. Recently, a series of studies have confirmed the latter idea, and several genera have been created to receive *Woloszynskia* species, known as woloszynskioids. Those genera have been distributed over different families: *Tovellia*, *Jadwigia*, *Esoptrodinium* and *Opisthoaulax* in the new family Tovelliaceae; *Borghiella* and *Baldinia* in the new family Borghiellaceae; *Biecheleria* and *Biecheleriopsis* ranged with the family Suessiaceae. These taxonomic changes have been supported by molecular data and by morphological differences in eyespot structure, organization of the cell apex and type of resting cyst.

Taxonomic knowledge about the diversity and geographic distribution of freshwater dinoflagellates in continental Portugal were assembled in a checklist for the first time and here presented (Chapter 2). Entries in the list were defined taking into account recent phylogenetic research, particularly the resulting taxonomic changes that affect genus-level limits of taxa. Published reports of freshwater dinoflagellate species, taken from 37 references, form the basis of the inventory, to which it was added documentation for 12 previously unreported taxa (11 species and one form).

Two new woloszynskioid species for science, not included in this checklist, are presented here (Chapter 3, 4). The morphology of cells and cysts is described as well as the ultrastructure of motile cells and particular aspects of the life cycle. LSU rDNA-based phylogenies confirm the new species. The first one belongs to the family Tovelliaceae, genus *Tovellia* (Chapter 3). The species epithet chosen was *aveirensis*, which constitutes a reference to the name of the university as well as the city where it has been found: University of Aveiro Campus, Aveiro, Portugal. *Tovellia aveirensis* has the peculiar feature of producing a resting cyst with paracingulum and ornamented by numerous branched processes, which not only differs from the bipolar and almost not ornamented *Tovellia* cyst, but also from all others described for woloszynskioids. Morphologically, it differs from other species of the genus

abstract (continuation)

mainly by having a line of knobs placed at the posterior edge of the cingulum, surrounding the cell, and lacking a distinct antapical plate around which the series of plates on the hypocone could be arranged.
The second new woloszynskioid has been found in a flooded area in Ribeiro da Palha stream, Nariz, Aveiro, Portugal, and in a freshwater pond in Scotland (Chapter 4). It belongs to the family Borghiellaceae, genus *Borghiella*, and was named *B. andersenii* in honor of Prof. Robert A. Andersen, who first established a culture of this species from material collected in Scotland. Morphologically, it is identical to *B. dodgei*, diverging from this mainly by having a rounded epicone and a shorter pair of elongate amphiesmal vesicles (PEV) with fewer knobs and lined on each side by two to three apical plates. *B. andersenii* is able to reproduce asexually both in the motile stage, by fission, and non-motile stage, with production of division cysts, something that has never been reported within Borghiellaceae so far. Furthermore, stronger evidences of sexual reproduction for this family have yet been observed in *B. andersenii* cultures, namely planozygotes and apparent resting cysts.
Two other woloszynskioids, designated as MSP1 and MSP12, are here briefly described (Chapter 5). These have been collected respectively in a farm pond at Gafanha da Boavista, near Vista Alegre, Ílhavo, Aveiro, and in the same place where *B. andersenni* was found. Both morphologic and phylogenetic results suggest that they are two new *Tovellia* species, evolutionarily close to *T. aveirensis*.

This thesis includes the following articles:

Pandeirada, M.S., Craveiro, S.C. & Calado, A.J. 2013. Freshwater dinoflagellates in Portugal (W Iberia): a critical checklist and new observations. *Nova Hedwigia* 97: 321–348 (DOI: 10.1127/0029-5035/2013/0119) – Chapter 2

Pandeirada, M.S., Craveiro, S.C., Daugbjerg, N., Moestrup, Ø. & Calado, A.J. Studies on woloszynskioid dinoflagellates VI: description of *Tovellia aveirensis* sp. nov. (Dinophyceae), a new species of Tovelliaceae with spiny cysts. *European Journal of Phycology* (submitted on 7 November 2013) – Chapter 3 – Inclusion in this thesis is not intended as effective publication of the new taxon proposed in the manuscript.

Daugbjerg, N., Andreasen, T., Happel, E., Pandeirada, M.S., Hansen, G., Craveiro, S.C., Calado, A.J. & Moestrup, Ø. . Studies on woloszynskioid dinoflagellates VII. Description of *Borghiella andersenii* sp. nov.: light and electron microscopy and phylogeny based on LSU rDNA. *European Journal of Phycology* (in preparation) – Chapter 4 – Inclusion in this thesis is not intended as effective publication of the new taxon proposed in the manuscript.

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LIST OF ABBREVIATIONS

AAl, Alto Alentejo

Ag, Algarve

ALP, apical line of narrow plates

av, amphiesmal vesicle

BA, bayesian analysis

BA, Beira Alta

BAl, Baixo Alentejo

BB, Beira Baixa

BB, Beira Litoral

BS, bootstrap support

bp, base pairs

C/c, chloroplast lobe

cc, collecting chamber

PCR, polymerase chain reaction

cob, mitochondrial cytochrome b

cox 1, mithocondrial cytochrome c oxidase 1

DL, Douro Litoral

E/e, eyespot

E, Estremadura

EAV, elongate apical vesicle

ivm, inner amphiesmal vesicle membrane

ITS, internal transcribes spacers

LF/lf, longitudinal flagellum

LM, light microscopy

LMR/r1, longitudinal microtubular root

LSU rRNA, large subunit ribosomal RNA (the 28S strand)

Mi, Minho

ML, maximum likelihood analysis

MSPn, number (n) of a certain culture line defined by Mariana Sofia Pandeirada

N/n, nucleus

nu, nucleolus

o, oil droplet

ovm, outer amphiesmal vesicle membrane

pc, postcincular plates

PEV, pair of elongate amphiesmal vesicles

pm, plasma membrane

Po, apical pore

pp, posterior probabilities

ps, pusular system

Py, central pyrenoid complex

R, Ribatejo

rDNA, nuclear DNA sequence that codes
for ribosomal RNA subunits

rRNA, ribosomal RNA

s, starch

SEM, scanning electron microscopy

sp, sulcal plate

SSU rRNA, small subunit ribosomal RNA
(the 18S strand)

T/t, trichocyst

TEM, transmission electron microscopy

TF/tf, transverse flagellum

TM, Trás-os-Montes e Alto Douro

vr, ventral ridge

x, canal plate

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CHAPTER 1

INTRODUCTION

GENERAL CHARACTERIZATION OF DINOFLAGELLATES

The dinoflagellates are a group of protists currently classified in the Alveolata, together with the ciliates and apicomplexans. These organisms are united morphologically by the presence of cortical alveoli underlying the plasma membrane (Baldauf 2008). The closest relatives of the alveolates are the Stramenopila (Heterokontophyta), a very diverse group that includes the macroscopic brown algae, the chrysophytes, the xanthophytes, the dictyochophytes, the raphidophytes, the pelagophytes and the diatoms, plus some traditionally non-algal groups such as the oomycetes (Adl et al. 2012).

Dinoflagellates comprise approximately 2000 extant and a similar number of extinct species, of which about 80% are marine and 20% inhabit freshwater habitats. Most of them are free-living, but they can also be parasitic (e.g., in copepods) or symbiotic (e.g., in corals) (Moestrup & Daugbjerg 2007; Fensome et al. 1993).

Dinoflagellate classification is traditionally based on the study of their external morphology through light microscopy (LM) and scanning electron microscopy (SEM) observations. LM has been determinant on the development of dinoflagellate taxonomy since the publication of the first identifiable descriptions of these microorganisms, in the late 1700s (Müller 1773, 1786). However, the visibility of small structures with this technique is limited and that can lead to misinterpretations. Such limitation has been overcome by the utilization of SEM, which was introduced to dinoflagellate studies in the 1960s (Lewis & Dodge 1990). Its generalized use has led to a more complete re-evaluation and description of many new taxa (Craveiro 2010).

In typical motile cells of dinoflagellates there are two heteromorphic flagella: the transverse flagellum (TF) usually in a transverse groove (the cingulum) that divides the cell in an apical (episome or epicone) and an antapical part (hyposome or hypocone); and the longitudinal flagellum (LF) inserted in a ventral-antapical groove called the sulcus (Fig. 1). An exception is found in the Prorocentrales, with both flagella inserted apically and without grooves (Fig. 2) (Popovský & Pfiester 1990; van den Hoek et al. 1995).

The outer region of dinoflagellate cells, designated as amphiesma, is constituted by a single layer of flat vesicles (amphiesmal vesicles) underlying the plasmalemma (van den Hoek et al. 1995). In the so-called armoured or thecate dinoflagellates, the amphiesmal vesicles contain more or less thick plates made of cellulose or a related polysaccharide (Fig. 3, a and c). In addition, armoured species which replace the amphiesma during cell

division or through ecdysis are, at least temporarily, surrounded by a thin additional layer, known as pellicle, underlying the amphiesmal plates. The pellicle contains cellulose and a sporopollenin-like substance, also found in the walls of resting zygotes of dinoflagellates, which is highly resistant to strong acids and bases (Popovský & Pfiester 1990; van den Hoek et al. 1995). *Ceratium* Schrank, *Peridinium* Ehrenberg, *Peridiniopsis* Lemmermann and the more recently described genus *Palatinus* Craveiro, Calado, Daugbjerg & Moestrup are examples of genera with thecate species (Popovský & Pfiester 1990; Craveiro et al. 2009).

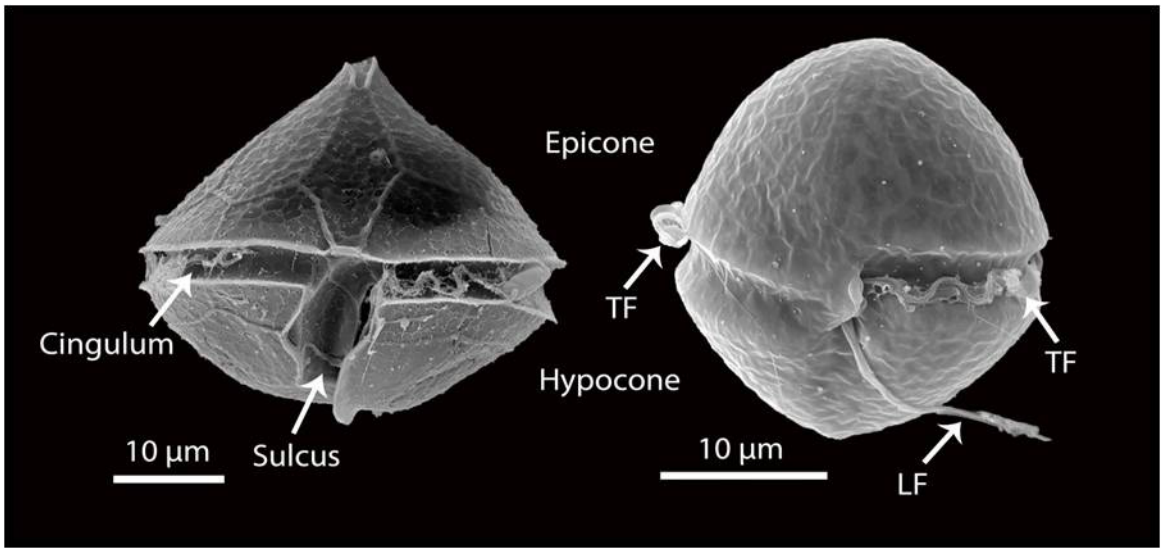

Fig. 1. SEM of *Diplopsalis acuta* (Apstein) Entz (left) and *Tovellia* sp. (right). Ventral views showing the general external morphology. The cingulum, which divides the cell in an apical part (epicone) and an antapical part (hypocone), and the sulcus are both deeply incised in *D. acuta*. The transverse flagellum (TF) in the cingulum and the longitudinal flagellum (LF) in the sulcus are marked in *Tovellia* sp.. Original.

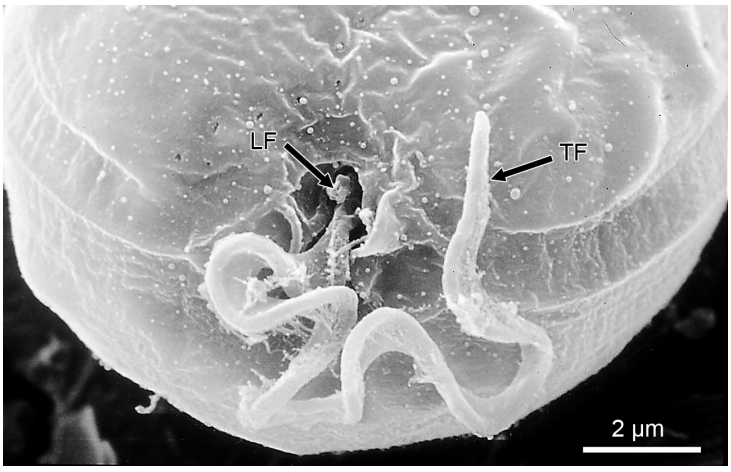

Fig. 2. SEM of *Prorocentrum cassubicum* (Wołoszyńska) Dodge, a dinoflagelate without cingulum or sulcus. Apical view showing the transverse flagellum (TF) and the stub of the longitudinal flagellum (LF). Original.

In athecate or naked dinoflagellates amphiesmal plates are very thin or even absent (Fig. 3, b and d). Athecate dinoflagellates can be found in genera like *Katodinium* Fott, *Amphidinium* Claparède & J.Lachmann, *Baldinia* Gert Hansen & Daugbjerg and *Gymnodinium* F.Stein (Popovský & Pfiester 1990; van den Hoek et al. 1995; Hansen et al. 2007). The latter genus has been erected by Stein (1878) to hold all athecate dinoflagellates initially integrated in the thecate *Peridinium*. However, during the 20th century it was demonstrated that the cell cover of some *Gymnodinium* species included numerous thin amphiesmal plates (Wołoszyńska 1917; Biecheler 1952). Former *Gymnodinium* species with predominantly hexagonal amphiesmal vesicles containing thin plates were segregated into a separate genus, *Woloszynskia* R.H.Thompson (1951). Species of *Woloszynskia*, as defined by Thompson (1951), are currently known as woloszynskioids (see Fig. 4).

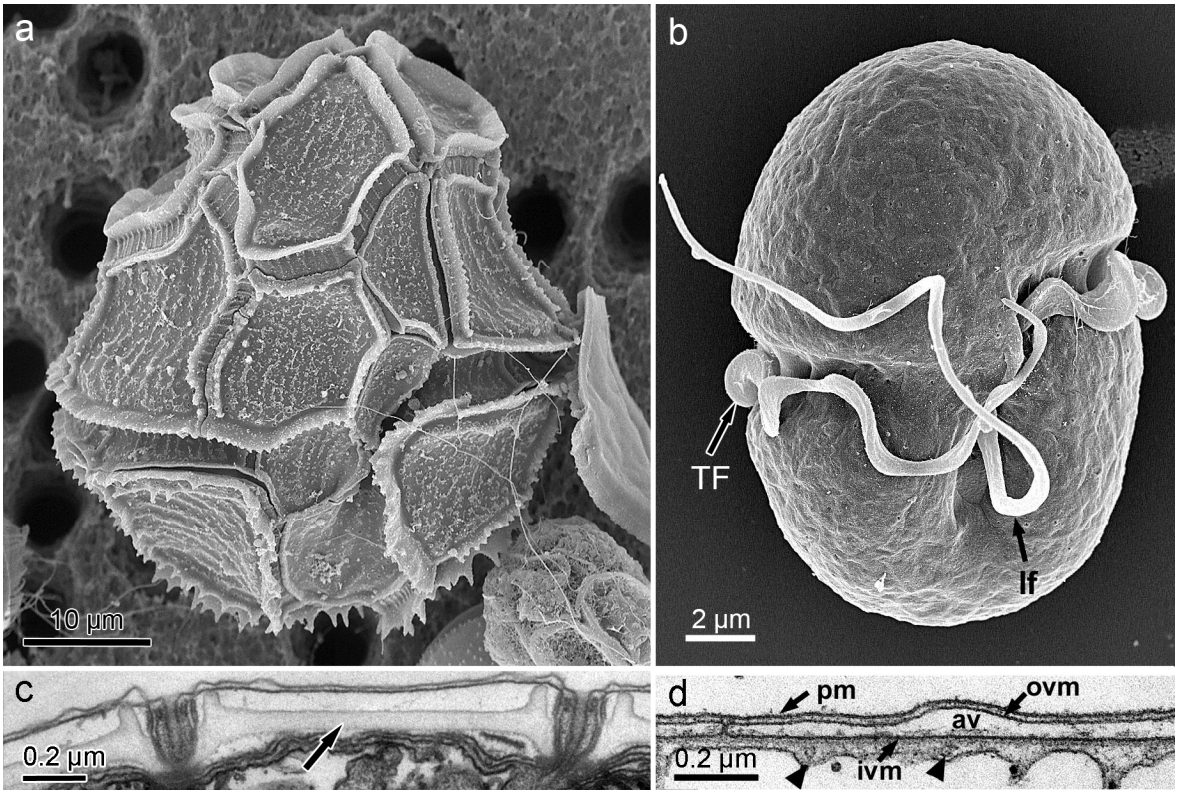

Fig. 3. Thecate and athecate dinoflagelates. (a) SEM of *Palatinus apiculatus* (Ehrenberg) Craveiro, Calado, Daugbjerg & Moestrup with strongly ornamented raised borders of thick plates. (b). SEM of *Baldinia anauniensis* Gert Hansen & Daugbjerg showing the transverse (TF) and longitudinal (lf) flagella. (c) The amphiesma of *P. apiculatus*, TEM. Thick cellulosic plate inside the amphiesmal vesicle (arrow) (d) Amphiesma of *B. anauniensis*, TEM. av, amphiesmal vesicle; ivm, inner amphiesmal vesicle membrane; ovm, outer amphiesmal vesicle membrane; pm, plasma membrane; arrowheads point to subthecal microtubules. (a) Original. (b) and (d) adapted from Hansen et al. 2007; (c) adapted from Craveiro et al. 2009.

Some gymnodinioid (i.e., naked) and woloszynskioid dinoflagellates have a straight or slightly curved apical 'line' on the epicone surface, extending over the anterior end from the ventral to the dorsal side of the cell (Fig. 4a) (Lindberg et al. 2005). This structure has been variously called carina (e.g., Thompson 1951) or acrobase (e.g., Roberts et al. 1995) and it is now considered an important character at the generic level (Daugbjerg et al. 2000; Moestrup & Daugbjerg 2007). Seven different organizations of apical complexes are currently recognized in dinoflagellates, three of them found in woloszynskioids (see below, in the introductory part to the group) (Moestrup & Daugbjerg 2007; Moestrup et al. 2008).

One of the most distinctive intracellular features of dinoflagellates is the nucleus, designated as dinokaryon (Fig. 5a). The chromosomes remain condensed throughout the mitotic cycle, without histones binding the DNA (and therefore without nucleosomes), and dividing through a closed mitosis. In addition, dinoflagellates have some of the largest known nuclear genomes, with considerable amounts of repetitive DNA (van den Hoek et al. 1995; Moestrup & Daugbjerg 2007; Baldauf 2008). According to Hackett et al. (2005), the repetitive DNA has perhaps structural importance, compensating the lack of histones.

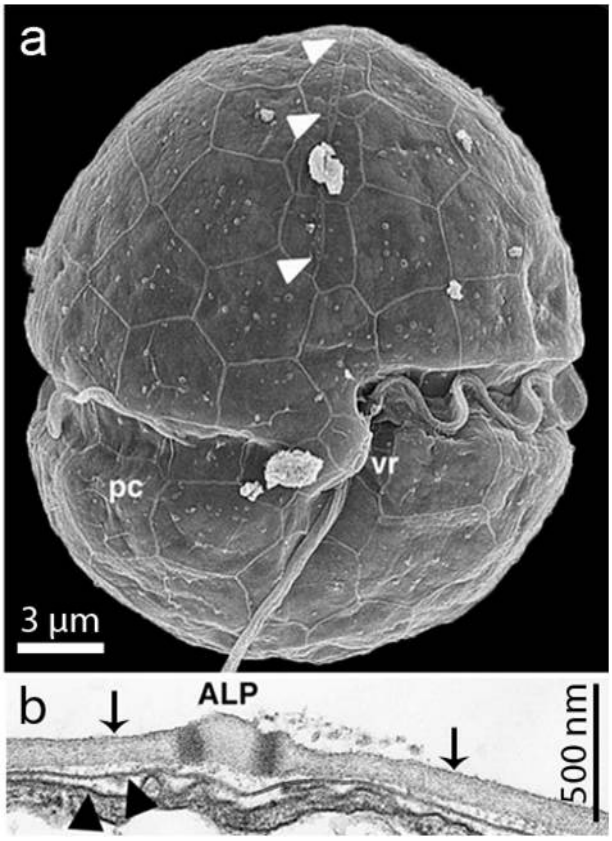

Fig. 4. *Tovellia coronata* (Wołoszyńska) Moestrup, Lindberg & Daugbjerg, a woloszynskioid. (a) SEM of *T. coronata*. pc, postcingular plates; vr, ventral ridge; the arrows indicate the apical line of narrow plates ALP. (b) The amphiesma of *T. coronata* showing an ALP section, TEM. The cell is bordered by amphiesmal plates (arrows) that lack the outer membrane. The plates are underlain by two distinct membranes (arrowheads). Adapted from Lindberg et al. (2005).

About half of the described dinoflagellate species has chloroplasts (Fig. 5a). The other half lacks chloroplasts and is exclusively heterotrophic. Some species with chloroplasts are able to combine the photosynthetic capability with heterotrophy and are called mixotrophic (Hansen & Calado 1999; Moestrup & Daugbjerg 2007).

The most common type of dinoflagellate chloroplast is bounded by three membranes, contains thylakoids mainly in groups of three, has chlorophylls a and c and peridinin as the major xanthophyll (Horiguchi 2004; Keeling 2004). These chloroplasts have evolved from red algal chloroplasts acquired through secondary endosymbiotic events and are known as chloroplasts type 1 (Moestrup & Daugbjerg 2007).

According to Moestrup & Daugbjerg (2007), there are eight types of chloroplasts in dinoflagellates that result from different endosymbiotic events. Some well-known examples are the presence of cryptophycean chloroplasts (chloroplasts type 6) in *Gymnodinium aeruginosum* F.Stein (Schnepf et al. 1989) and chloroplasts with a diatom origin (chloroplast type 7) in *Durinskia baltica* (Levander) Carty & El.R.Cox and *Kryptoperidinium foliaceum* (F.Stein) Er.Lindemann (Horiguchi 2004).

The principal storage product in dinoflagellates is starch, which is synthesized outside the chloroplasts. Lipid is also found in the form of globules and droplets (van den Hoek et al. 1995).

The eyespot (Fig. 5, a and b), an organelle related to light reception, is present in some species, associated with the longitudinal microtubular root (LMR = root r1) in the sulcal area (Moestrup et al. 2008). Currently, six types of eyespots are recognized in dinoflagellates, which were designated as types A to F (Moestrup & Daugbjerg 2007; Craveiro et al. 2010). Three of them (types B, C and E) are found in woloszynskioids and are described below, in the introductory part dedicated to the group.

The structure of the eyespot is highly conserved in most algal groups and is usually shared by all members of an algal class or even phylum (Moestrup et al. 2008); it is therefore expected to be an important phylogenetic marker. The assertion that different types of eyespots correspond to different lineages of dinoflagellates has been supported by molecular data (e.g., Moestrup & Daugbjerg 2007) and constituted the basis for the reorganization of species groups of woloszynskioids.

The pusule (Fig. 5, a and c) is an organelle typical of dinoflagellates and it is believed to have a function of excretion and osmoregulation (Dodge 1972; Moestrup & Daugbjerg

2007). It is formed by a system of apparently empty tubes, or vesicles, with a close association between their bounding membrane and the inner membrane of an enveloping vesicle (van den Hoek et al. 1995; Craveiro 2010). Pusules do not contract, unlike the contractile vacuoles found in many other flagellates, especially those living in freshwater (Popovský & Pfiester 1990; van den Hoek et al. 1995). Based on his revision of the types of pusules present in 40 freshwater and marine dinoflagellates, Dodge (1972) classified them into seven types, included in the following two categories: (1) pusules with vesicles that connected directly to the flagellar canal, or to collecting chambers, or to pusular tubes; (2) pusules constructed of tubules or sacks only, with or without invaginated portions.

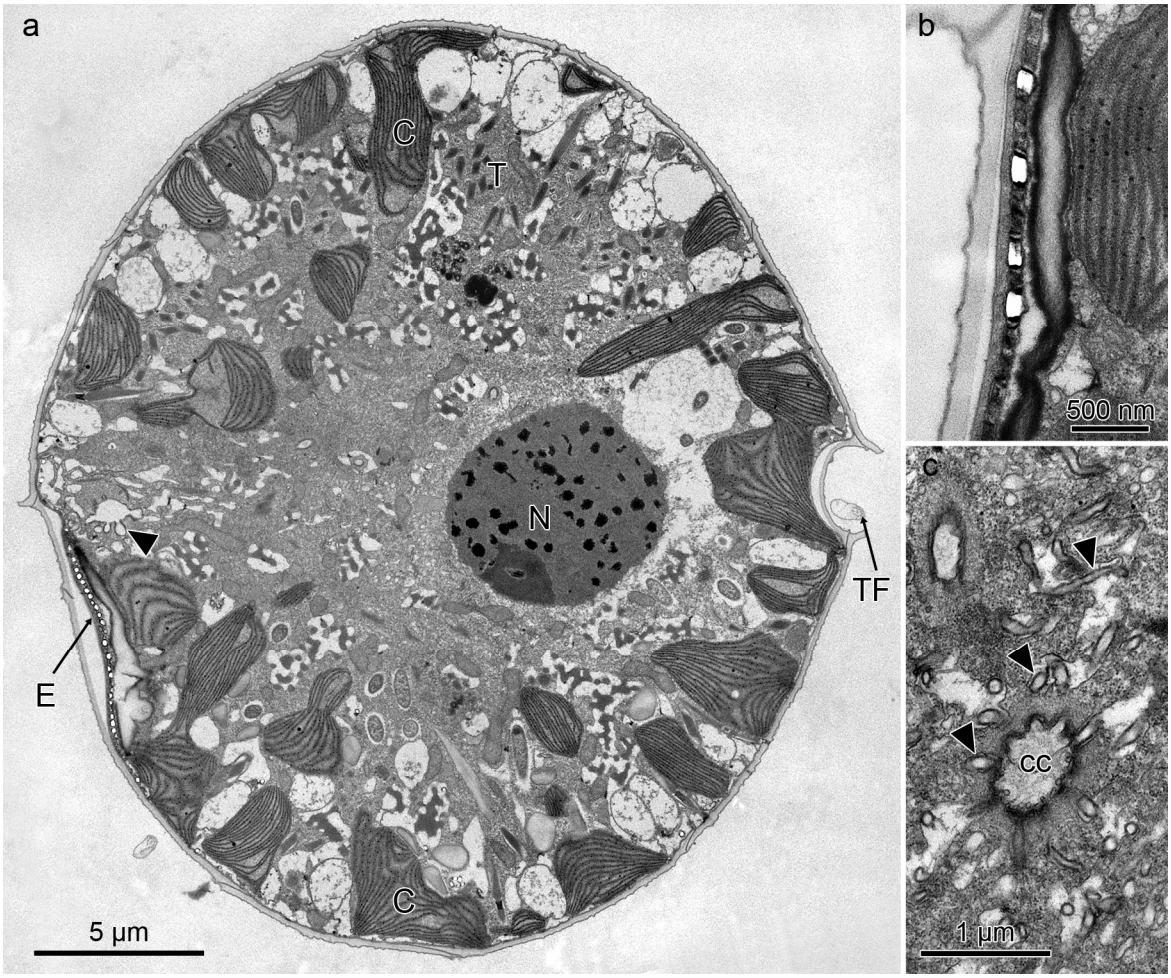

Fig. 5. Ultrastructure (TEM) of *Sphaerodinium cracoviense* Wołoszyńska. (a) Longitudinal section of the cell seen from the left side showing some of the typical features of dinoflagellates: the cingulum, seen on the dorsal side, with the transverse flagellum (TF), nucleus (N), trichoysts (T), chloroplast lobes (C) and the eyespot (E) in the sulcal area. The arrowhead points to pusular vesicles. (b) Higher magnification of eyespot with a layer of oil and a layer of brick-like components. (c) Pusular system with several pusular tubules (arrowheads) that connect to a collecting chamber (cc). Original.

MOLECULAR TOOLS IN DINOFLAGELLATE CLASSIFICATION

Molecular studies had their great development in the past 20 years, mainly through the elaboration of phylogenies based on sequences of nuclear DNA that code for ribosomal RNA subunits and intervening spacers (designated as rDNA) (Logares 2007). The sequences of rDNA have the advantage to be widespread in eukaryotes, with its four components involved in eukaryotic ribossome constitution: the 18S (small subunit, SSU), the 28S (large subunit, LSU) and the 5.8S, together with two internal transcribed spacers (ITS1 and ITS2), are included in a single pre-rRNA transcription unit; the 5S fragment is coded in other regions of the genome. The ITS sequences are less conservative and may be used for a different level of discrimination. Another advantage is the presence of many rDNA sequences in the genome, which means there is abundant template for genetic amplification (Craveiro 2010).

Mitochondrial cytochrome b (cob), mithocondrial cytochrome c oxidase 1 (cox 1), HSP90, actin, alpha- and beta-tubulin gene sequences are examples of other phylogenetic markers used (Leander & Keeling 2004; Lin et al. 2002; Saldarriaga et al. 2004; Zhang et al. 2005).

Molecular studies have led to great changes in our understanding of the phylogenetic relationships of dinoflagellates. "Max" Taylor (1980) presented a phylogenetic tree with most heterotrophic genera placed at the top of the tree and photosynthetic species occupying many of the basal branches. However, molecular evidence (e.g., Saldarriaga et al. 2004) has turned the tree over and its base is now occupied by heterotrophic species only, some free living and some parasitic (Moestrup & Daugbjerg 2007). *Noctiluca scintillans* (Macartney) Kofoid is an example of heterotrophic species placed at the base of tree taking into account SSU data (Saldarriaga et al. 2004), the same data that had already suggested the genus *Noctiluca* Suriray as the earliest diverging dinoflagellate lineage (Saunders et al. 1997). Saldarriaga et al. (2004), based on SSU and LSU rDNA, also indicated that amphiesmal plates have been lost repeatedly during dinoflagellate evolution.

Several recent works have led to changes mainly at generic level, with special relevance to the “naked” species. An example is the description of the three new genera *Karenia* Gert Hansen & Moestrup, *Karlodinium* J.Larsen and *Akashiwo* Gert Hansen & Moestrup, and the redefinition of the genus *Gymnodinium* based on morphological

features, supported by molecular information obtained from LSU rDNA partial sequences (Daugbjerg et al. 2000).

Woloszynskioid species have also been revised on the basis of ultrastructure and LSU rDNA phylogenies leading to their redistribution into new genera and families (see below).

ASPECTS OF LIFE CYCLE AND THEIR RELEVANCE FOR DINOFLAGELLATE CLASSIFICATION

The knowledge of external and internal features of cells and molecular data are crucial to identify and classify dinoflagellates. However, there are other important aspects within their life cycle that can be also determinant for taxonomic decisions, such as the type of resting cyst produced (Moestrup & Daugbjerg 2007).

The life cycle of most dinoflagellates is interpreted as haplontic, with only the zygote nucleus being diploid (Popovský & Pfiester 1990; van den Hoek et al. 1995). According to Popovský & Pfiester (1990), their life history can be divided into vegetative and reproductive phases. During the former, young cells grow and mature until they divide, producing more vegetative cells (asexual reproduction), thus increasing the population. In case of unfavorable conditions, the new cells obtained from division can act as gametes. At the end of the growth season a resting cyst is usually produced, often after the occurrence of gamete fusion (sexual reproduction). When the conditions become favorable the cyst may germinate and produce vegetative cells.

Cell division is often a bipartition, with each of the new cells receiving half the parent amphiesma and forming the missing part of the armour, as in species of *Ceratium* Schrank, (Fig. 6, a and b) (van den Hoek et al. 1995; Moestrup & Daugbjerg 2007). The division can also occur when the cell is in a non-motile stage, a so-called temporary or division cyst (Fig. 6c). This happens in genera like the thecate *Peridinium* and *Peridiniopsis*. In this case, the flagella are lost and the dinokaryon divides together with the cytoplasm into two equal parts within the parent theca. After a short period, some plates of the parent theca disconnect, or the epi- and hypocone separate, releasing the gymnodinioid (i.e., naked, at least in the initial stages) daughter cells (Popovský & Pfiester 1990).

Some dinoflagellate species are able to divide in both motile and non-motile stages. In species of *Gymnodinium* can occur the oblique division of motile cells into two daughter cells or the production of autospores (non-motile cells) from non-motile cells surrounded by layers of mucilage. In woloszynskioids, it is described the division of motile or non-

motile cells into two daughter cells as well as the division of the protoplast within the parent cell cover originating as many as 8 daughters cells (Popovský & Pfiester 1990).

Fig. 6. LM of motile (a, b) and non-motile division stages (c). (a, b) Cell division by oblique binary fission in *Ceratium furcoides* (Levander) Langhans. Both images with same scale. (c) Dividing *Peridinium cinctum* (O.F. Müller) Ehrenberg in the parent theca. (a) and (b) Original. (c) Adapted from Lefèvre 1932.

Sexual reproduction normally occurs by isogamy or anisogamy, often followed by the formation of a resting cyst (Moestrup & Daugbjerg 2007). This process is especially important because it allows for continuous adaptation in a changing environment due to genetic recombination, DNA repair and avoidance of unfavorable mutations (Figueroa et al. 2010).

When environmental conditions are not favourable for population growth, cells resulting from division can act as gametes which tend to have few chloroplasts, numerous membrane-bound accumulation bodies, many starch grains, and chromosomes that appear slightly unwound (Popovský & Pfiester 1990).

In the laboratory, sexual reproduction can be induced when exponentially growing cells are inoculated into nutrient deficient media, mainly missing nitrogen or phosphates, and/or exposed to short light periods and/or unfavorable temperatures (Hickel 1988; Popovský & Pfiester 1990; Figueroa et al. 2005; Figueroa et al. 2011). The sexual process can occur either through fusion of genetically identical gametes (self-fertilization or homothalism) or by fusion of gametes from genetically different strains (outcrossing or heterothallism) (Figueroa et al. 2010). Some mechanisms of gamete fusion described are: wall-to-wall adhesions through cingular, basal and apical regions, and with cingular-apical joint (Fig. 7, a and b) (Figueroa & Bravo 2005); connection of the sulcal region of the

gametes at different angles of their cingula, ranging from a perpendicular to an almost parallel orientation (Fig. 7c) (Figueroa et al. 2009); mating through flagellar attachment (Figueroa & Bravo 2005); mating through the formation of a fertilization tube (Fig. 7d) (Spector et al. 1981; Bhaud et al. 1988); and fusion through incorporation of the male gamete by the female (Fig. 8, a, b and c) (Hickel 1988).

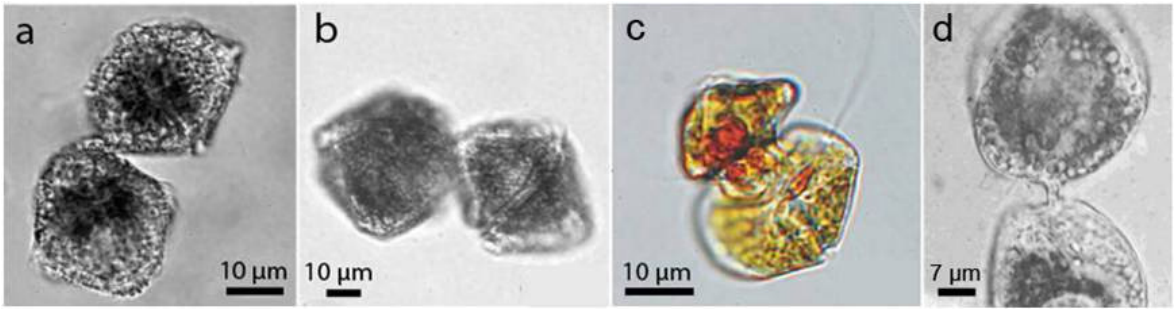

Fig. 7. Mechanisms of gametes fusion (LM). Fusing pairs of *Lingulodinium polyedrum* (F.Stein) Dodge (a, b), *Kryptoperidinium foliaceum* (c), and (d) *Prorocentrum micans* Ehrenberg (d). Wall-to-wall contact through basal regions (a) and cingular-apical joint (b). Attachments through the sulcal region (c) and fertilization tube (d). (a) and (b) Adapted from Figueroa & Bravo 2005; (c) Adapted from Figueroa et al. 2009; (d) Adapted from Bhaud et al. 1988.

After cell fusion a diploid zygote is formed, known as planozygote, which approximately resembles the typical vegetative cell, but has two longitudinal flagella (Fig. 8b) (Popovský & Pfiester 1990). In some dinoflagellate species, the planozygote develops into a resting cyst, called hypnozygote, which contains extremely resistant sporopollenin-like material in its wall contributing to an higher resistance to extreme environmental changes (Fig. 8d) (van den Hoek et al. 1995; Figueroa et al. 2010; Mertens et al. 2012).

The hypnozygote undergoes a period of dormancy controlled by internal and external factors. A minimum time is necessary for internal maturation, the mandatory dormancy period, which is not influenced by external conditions. These conditions are mostly determinant of the germination time (Figueroa & Bravo 2005). In *Peridiidium cinctum,* the period of dormancy reaches approximately three months at optimal culture conditions at 20ºC and about five months at 4ºC both in light and dark conditions (Popovský & Pfiester 1990).

In general, germination is inhibited by low temperature, anaerobiosis, salinity, darkness and nutritional deficiency (Figueroa & Bravo 2005; Figueroa et al. 2005).

Nevertheless, the exposure of cysts to low temperatures (about 4 ºC) for several weeks may induce their germination (Popovský & Pfiester 1990). In marine species of *Scrippsiella* Balech *ex* A.R.Loeblich III and *Alexandrium* Halim germination is promoted by decreasing salinity and increasing nutrient concentration (Figueroa et al. 2005).

During the germination usually occurs the loss of part of, or an opening in, the cyst wall, termed archeopyle (Mertens et al. 2012). Normally, one cell with two longitudinal flagella, called planomeiocyte, germinates from the cyst and undergoes meiosis to re-establish the haploid vegetative phase (Popovský & Pfiester 1990; Figueroa & Bravo 2005; Figueroa et al. 2005). There are reports of up to four planomeiocytes released (Popovský & Pfiester 1990) and some of them with a single longitudinal flagellum, as in *Kryptoperidinium foliaceum* (Figueroa et al. 2005). The meiosis can also occur in the planozygote, thus skipping the encystment (Figueroa & Bravo 2005), or in the hypnozygote (Pfiester 1976). In some species the first meiotic division is preceded by nuclear cyclosis, a swirling of the chromosomes within the nuclear envelope (e.g. Stosch 1973).

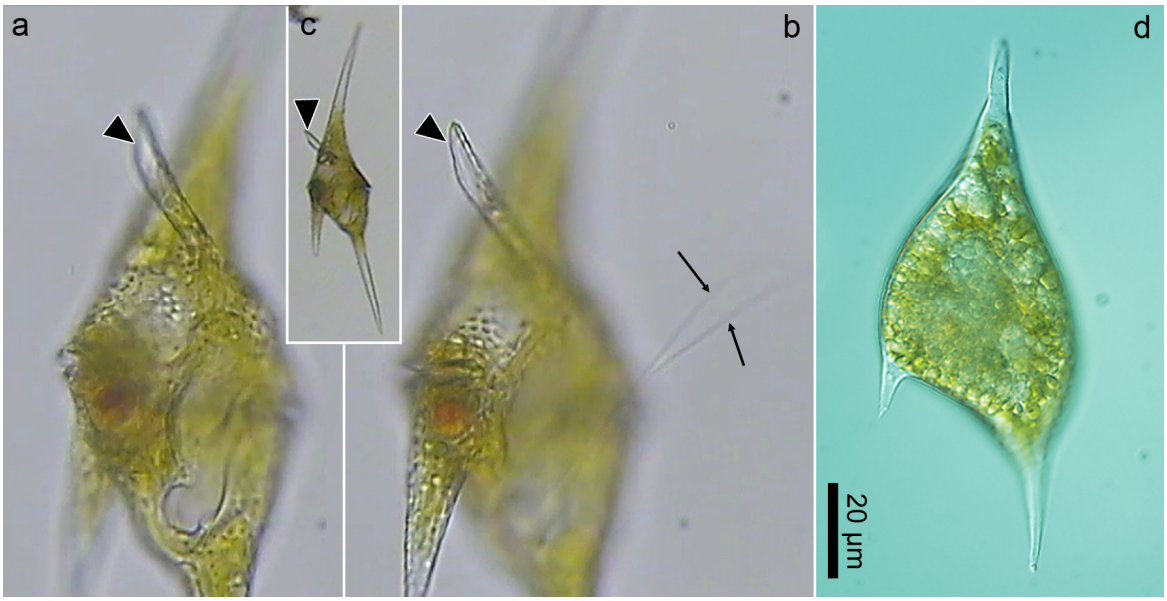

Fig. 8. LM of *Ceratium furcoides*. (a, b) Ventral view of late stage of fusion of gametes with almost complete incorporation of the male gamete (arrowhead). Both longitudinal flagella are marked in (b) with arrows. (c) Dorsal view of same stage of fusion. (d) Mature hypnozigote. (a), (b) and (d), same scale. Original.

WOLOSZYNSKIA R.H.THOMPSON: TAXONOMIC ISSUES AND REVISION

Dinoflagellates known as woloszynskioids are mostly those that have been integrated at some stage in the genus *Woloszynskia* R.H.Thompson for being covered with numerous thin amphiesmal plates, a feature that separated them from the athecate *Gymnodinium*. Nevertheless, *Woloszynskia* has been the subject of some controversy mainly associated with the establishment of its type species and with the polyphyly of its species (Lindberg et al. 2005).

Thompson's (1951) original description of *Woloszynskia* did not indicate a type species. *Woloszynskia reticulata* R.H.Thompson was later designated by Loeblich Jr. & Loeblich III (1966) as type of *Woloszynskia*. This was an inconvenient choice insofar as this species is morphologically different from all others inserted in the genus by having a hypocone covered with prominent, thick and concave plates (Fig. 9) (Lindberg et al. 2005; Moestrup et al. 2008).

The first indications that *Woloszynskia* was polyphyletic came from the different types of resting cysts and eyespots found in the different species. Stosch (1973) suggested that the genus would comprise three subgroups taking into account the three different resting cysts found in woloszynskioids: round to oval and smooth-walled; round to oval with numerous spines (or bristles); and slightly elongate with paracingulum and two axial horns, in addition to lateral protuberances or scattered, short, thick spines (Fig. 10) (Stosch 1973; Lindberg et al. 2005). In the 1960s and 1970s, John Dodge and co-workers demonstrated in TEM different types of eyespots in *Woloszynskia coronata* and *Woloszynskia tenuissima* (Lauterborn) R.H.Thompson (Crawford & Dodge 1971; Crawford et al. 1971). More recently, Kremp et al. (2005) illustrated a third type in the so-called *Woloszynskia halophila* (Biecheler) Elbrächter & Kremp.

Recently, several studies have confirmed that *Woloszynskia* is polyphyletic and the genus has been divided into six new genera: *Tovellia* and *Jadwigia* included in the new family Tovelliaceae (Lindberg et al. 2005), *Borghiella* (Moestrup et al. 2008) and *Baldinia* (Hansen et al. 2007) included in the new family Borghiellaceae (Moestrup et al. 2009a), *Biecheleria* (Moestrup et al. 2009a) and *Biecheleriopsis* (Moestrup et al. 2009b) included in the family Suessiaceae. Such deep modification has taken into account differences in the eyespot and apical complex structures, the type of resting cyst produced and molecular data.

In addition to the redistribution of woloszynskioid species, these works had a special importance due to the description of two new apical complexes: the apical line of plates (ALP; Fig. 4) (Lindberg et al. 2005) and the pair of elongate amphiesmal vesicles (PEV) (Moestrup et al. 2008), which together with the elongate apical vesicle (EAV) constitute the three apical complexes presently known in these species (Moestrup & Daugbjerg 2007).

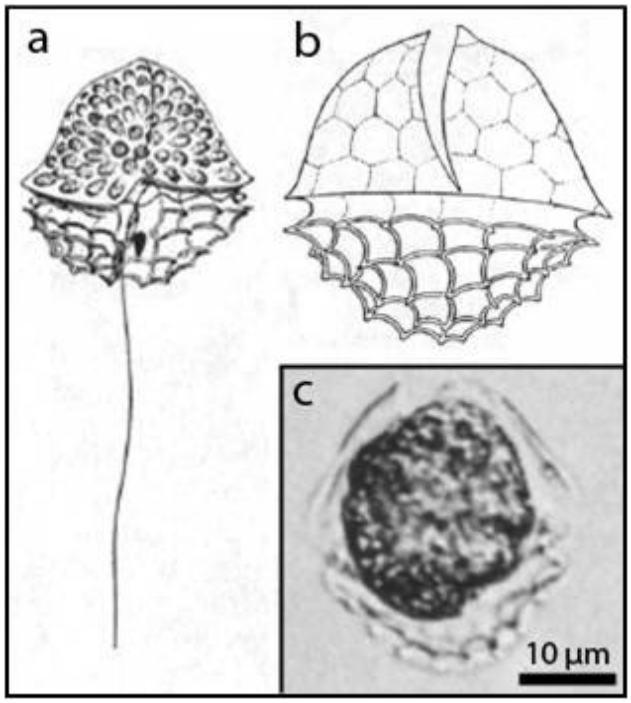

Fig. 9. *Woloszynskia reticulata*. (a) and (b) Ilustrations by Thompson (1951). (a) Ventral view of a young living cell. (b) Dorsal view of the theca. (c) LM of a vegetative cell with carina split. Adapted from Pfiester et al. (1980).

Woloszynskioid genera were divided into three groups, each corresponding to a different type of eyespot (Lindberg et al. 2005) and supported by molecular data (e.g., Moestrup & Daugbjerg 2007). Group I corresponds to the family Tovelliaceae, which includes freshwater dinoflagellates with eyespot type C (Moestrup & Daugbjerg 2007). This is characterized for being extraplastidial and composed of pigment globules not bounded by membranes. *Tovellia* and *Jadwigia* are two recent genera integrated in the Tovelliaceae and comprise species with an ALP (Lindberg et al. 2005). *Tovellia* species produce resting cysts with a paracingulum, two opposite axial horns and pre- and postcingular protuberances or scattered short spines (Fig. 10, a and b). *Jadwigia applanata* Moestrup, Lindberg & Daugbjerg, the only species presently included in *Jadwigia*, produces smooth, round resting cysts (Fig. 10c) (Lindberg et al. 2005; Moestrup et al. 2006). This family also comprises *Esoptrodinium* Javornický (Lindberg et al. 2005; Calado

et al. 2006) and the recently described genus *Opisthoaulax* Calado, created to receive some of the freshwater, phagotrophic species of *Katodinium* (Calado 2011).

Group II includes marine and freshwater dinoflagellates of the family Suessiaceae, which possess an eyespot of Moestrup & Daugbjerg's (2007) type E. This is formed by a stack of cisternae, each one containing brick-like material. *Biecheleria* and *Biecheleriopsis* are two recently described genera integrated in this group and comprise species with an EAV on the epicone (Moestrup et al. 2009a, 2009b). Round or oval cysts with numerous spines have been found in *Biecheleria pseudopalustris* (J.Schiller) Moestrup, K.Lindberg & Daugbjerg (Fig. 10d) (Moestrup et al. 2009a). Moestrup et al. (2009b) showed in Fig. 13 an oval structure with short spines as a possible cyst of *Biecheleriopsis adriatica* Moestrup, K.Lindberg & Daugbjerg. The genera *Symbiodinium* Freudenthal and *Polarella* Montresor, Procaccini & Stoecker, and the species *Protodinium simplex* Lohmann and *Prosoaulax lacustris* (F.Stein) Calado & Moestrup also have type E eyespots and are also included in the family Suessiaceae (Lindberg et al. 2005; Moestrup et al. 2009a).

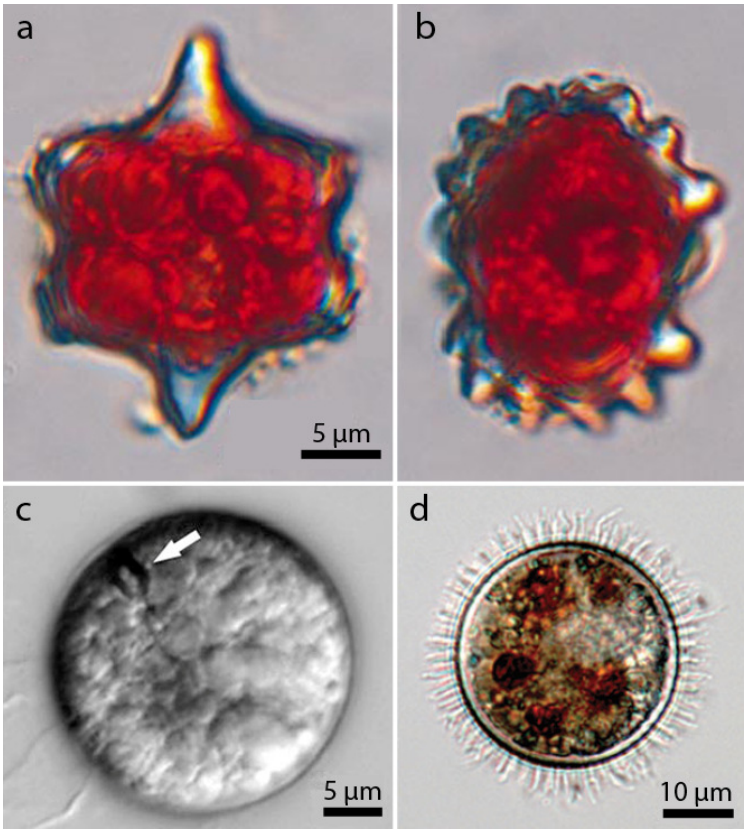

Fig. 10. Examples of resting cysts found in woloszynskioids, LM. (a) and (b) resting cysts of *Tovellia coronata*. The cyst is extended into two opposite axial horns (a). Both epicone and hypocone bear a row of lateral knobs (b). (c) spherical and smooth resting cyst of *Jadwigia applanata* Moestrup, Lindberg & Daugbjerg. The arrow indicates the eyespot. (d) resting cyst of *Biecheleria pseudopalustris* (J.Schiller) Moestrup, Lindberg & Daugbjerg. The cyst has a thick wall, dark reddish pigment spots and long bristles. (a), (b) and (c) adapted from Lindberg et al. (2005); (d) adapted from Moestrup et al. (2009a).

Group III corresponds to the recently described family Borghiellaceae, which includes freshwater species of *Borghiella* and *Baldinia* with an eyespot of type B (Moestrup & Daugbjerg 2007). This consists of intraplastidial oil globules and an overlying cover of vesicles containing brick-like material on the ventral side. *Borghiella* has a PEV, whereas no apical complex was detected in *Baldinia*. The cysts are smooth, spherical to oval in *Borghiella*, and more irregularly elongate with an axial invagination in *Baldinia* (Hansen et al. 2007; Moestrup et al. 2009a).

AIMS OF THE WORK

Taxonomic works with documented records dedicated to dinoflagellates present in Portuguese freshwaters are nonexistent. References to species of freshwater dinoflagellates are scattered over some three dozens of articles, of which some are concerned primarily with the general composition of the phytoplankton community of lagoons and reservoirs, while others address the identity of microalgae of all lineages, and a few concentrate on ultrastructural and phylogenetic aspects of selected species (full bibliographic data are provided in the next chapter). A total of about 35 species is cited in these works, with *Gymnodinium* and *Peridinium*, in their traditional, broad concepts, accounting for most of the taxa reported from Portugal. Nevertheless, recent revisions of taxonomic concepts require reassignment of a large fraction of these species to different genera. A predictable change will be the transference of the woloszynskioids indicated as members of *Gymnodinium* and *Gyrodinium* Kofoid & Swezy to the genus *Woloszynskia* or the recent genera created to hold several of them.

According to what was referred throughout the introduction, and from my own experience, woloszynskioids are a remarkably difficult group of dinoflagellates to identify. LM observations are often unsatisfactory for obtaining reliable identifications. Even with more advanced LM equipment it is difficult to clearly detect the very thin plates covering the cells, a determinant feature for classification within the group (e.g., Thompson 1951). Currently, such observations are made easier by using SEM techniques, which have become crucial in the identification and morphological description of woloszynskioids.

As seen before, *Woloszynskia sensu lato* is a polyphyletic genus, which comprises several taxonomic groups with the same general type of amphiesmal organization. The nature of the amphiesma, formerly use to characterize genera, is being replaced by

molecular and ultrastructural data, such as the type of eyespot, apical complex and resting cyst produced. The creation of new genera and families to hold woloszynkioids and related species led to the need to revise the taxonomic position of several dinoflagellates to evaluate their phylogenetic relationship relative to those included in these new groups. Eventually, other genera and families may emerge as a consequence.

Nowadays, the advanced microscopical and molecular techniques not only improve the conditions to study dinoflagellates but also create fascinating challenges, such as the study of woloszynskioids, a changing group in which the taxonomic position of several species needs to be re-evaluated. With this in mind, the main purpose of this work was to contribute to the knowledge of dinoflagellates, in particular of woloszynskioids, through their fine-characterization using a combination of LM, SEM and TEM techniques, and molecular approaches. More specifically, the aims were:

1. Elaboration of a checklist assembling all the information about the diversity and geographic distribution of freshwater dinoflagellates in continental Portugal taken from previously published material.
2. Collection in freshwater localities, mainly from Aveiro region, in order to know the dinoflagellate diversity, especially of woloszynskioids.
3. Isolating into culture all the potentially different woloszynskioids found during the sampling period.
4. Morphologic and ultrastructural characterization of the woloszynskioid species, including particular aspects of their life cycle.
5. Sequence determination of partial LSU rDNA from all woloszynskioids in culture and phylogenetic analysis for an easier discrimination of new species of this group of organisms.
6. Description of eventual taxonomic novelties in the woloszynskioids found.

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CHAPTER 2

FRESHWATER DINOFLAGELLATES IN PORTUGAL (W IBERIA): A CRITICAL CHECKLIST AND NEW OBSERVATIONS

A slightly abbreviated version of this chapter was published in:

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ABSTRACT

Current taxonomic knowledge about the diversity and geographic distribution of freshwater dinoflagellates in continental Portugal is assembled in a checklist for the first time. Entries in the list were defined taking into account recent phylogenetic research, particularly the resulting taxonomic changes that affect genus-level limits of taxa. Published reports of freshwater dinoflagellate species, taken from 37 references in this account, form the basis of the inventory, to which we add documentation for 12 previously unreported taxa (11 species and one form). Reported names are assembled into 51 entries, of which one we consider a *nomen dubium*, corresponding to 49 species and one form, representing 24 genera of dinoflagellates. The impact of recent taxonomic work is manifest in the assignment of 15 entries to 11 genera established within the last 15 years. The bases for our taxonomic decisions, especially when they differ from those adopted by available identification manuals, are outlined in brief notes that follow the relevant entries. Full bibliographic data are provided for all taxa mentioned in the checklist entries.

Key words: dinoflagellates, Dinophyceae, freshwater, light microscopy, Portugal, scanning electron microscopy

INTRODUCTION

Our knowledge about the occurrence of dinoflagellate species in the freshwater bodies of Portugal has never been collected into a single source. The present critical checklist is an attempt to do so within the framework of a taxonomy that has been extensively modified since the latest geographically comprehensive identification manual became available (Popovský & Pfiester 1990). The list is expected to be useful for the preparation of two forthcoming floristic treatments of freshwater dinoflagellates: one is a contribution to the series dealing with continental algae of the Iberian Peninsula, for which one volume has been published (Cirujano 2008); the other is a world flora of freshwater dinoflagellates that is currently in preparation for the Süßwasserflora von Mitteleuropa series by Øjvind Moestrup and one of the present authors (AJC).

The list was built from published records (see Materials and Methods for literature coverage) to which we added 12 taxa from our own observations. Photographic

documentation is given for all taxa previously unreported. Geographic coverage is limited to continental Portugal, i.e., the Macaronesian islands of the Azores and Madeira are not included.

Because the list includes an important number of species that have been transferred to recently described genera that may be unfamiliar to non-specialists in the group, and because numerous mistakes persist in dinoflagellate literature by being copied from indirect sources, we decided to be explicit in the taxonomic treatment, indicating the relationship between all names involved and giving full reference to original publication data, taken from the original works.

The main steps in the build up of our knowledge of freshwater dinoflagellates in Portugal, up to the time the present authors came into the scene, are briefly outlined. The earliest published record that we found of a dinoflagellate identified to species from Portuguese freshwaters dates from 1948. Its author, Francisco Soares de Lacerda, was among the first researchers in Portugal to extend Systematic Botany to the various groups of freshwater microscopic "plants", although he is mainly known for his work on the filamentous green algae of the family Oedogoniaceae (Lacerda 1946). In a country nearly devoid of natural lakes the construction of river dams introduced a new type of habitat: the reservoirs. In a series of papers published in the 1950's Fernando Frade Viegas da Costa (usually cited by the surname Frade), a zoologist in the University of Lisbon, addressed the changes in aquatic communities induced by the commissioning, in 1951, of the large Castelo do Bode reservoir, and gave the first reports of freshwater *Ceratium* (Frade 1954, 1957). However, the most notable contribution to the knowledge of Portuguese freshwater algae was due to Arnold Nauwerck, a Swedish researcher. Nauwerck identified and, to some extent, described the algae in samples fixed in Formalin or Lugol's solution, either sent to him by Manuel Póvoa dos Reis, first from near Coimbra (Nauwerck 1959) and later collected from lakes in the mountain Serra da Estrela in June 1960, or taken during Nauwerck's own visit to Portugal in the spring of 1960. Only rarely could he study live material due to the long travel times back to the laboratory in Coimbra. His systematic and ecological account of Portuguese planktonic algae (Nauwerck 1962) contains species lists from 18 locations, including 13 reservoirs, widely distributed from northern Minho to southern Alentejo. Póvoa dos Reis was himself a specialist in red algae who corresponded with foreign phycologists, among whom Henrichs Skuja, an outstanding and prolific

worker, celebrated by his detailed descriptions and exquisite representations of microalgae. Nauwerck was one of Skuja's students and the detailed accounts he gave of the organisms, notwithstanding the limitation of having only fixed material to work with, reveals mastery of the subject. Nauwerck's reports of dinoflagellates contribute to fundament 24 of the species entries in the present work.

The general taxonomic approach to the study of biodiversity followed by researchers in the Botanical Institute in Coimbra during the 1960's and 1970's (José Ernesto Mesquita Rodrigues, Jorge Almeida Rino, Maria de Fátima Santos) resulted in several new reports of dinoflagellates accompanied by descriptive data (Rodrigues 1961, Rino 1967, 1969, Santos 1976). The largest contributor to the list of works that cite freshwater dinoflagellates from Portugal is Maria do Rosário Leal de Oliveira, an hydrobiologist employed in official, state-owned laboratories, who conducted a large series of studies on the phytoplankton communities of reservoirs between 1976 (published under the surname Moita) and the late 1980's. Oliveira's studies were mainly ecological, especially oriented to the evaluation of productivity and general water quality of the reservoirs, and do not contain descriptions or illustrations of the species listed.

Our understanding of generic level relationships in dinoflagellates is developing rapidly. At the same time, specimens we cannot readily identify keep populating our samples. Therefore, it is expected, and hoped, that this checklist will soon become outdated.

MATERIALS AND METHODS

The checklist is a combination of previously published names and new additions. All publications dealing with freshwater environments in continental Portugal from which dinoflagellate species were reported were taken into account; these amount to 37 references in the present work. Mere citation of dinoflagellate genera or higher ranks was not considered for the list. Unpublished reports, theses and other so-called gray literature was not included, firstly because these are often not widely accessible (a situation that is changing rapidly with the growth of online bibliographic databases) and especially because they did not necessarily undergo a reviewing process that, in principle, published work had to get through. We systematically searched for dinoflagellate species reports in well over 150 publications dealing with freshwater algae from continental Portugal, collected by the

senior author during the last 25 years. The list is intended to be complete (i.e., we did not set a starting date as a limit), although it most certainly is not; we are well aware of how hopeless it is to try to cover the multitude of publications in journals and conference proceedings of all kinds. We will be grateful for any omissions that may be brought to our attention.

The new records of dinoflagellates for Portugal are the result of nearly 30 years of sampling, mostly in the Aveiro region (Fig. 1). In a more recent period, between October 2010 and June 2011, a regular, weekly sampling was conducted in some ponds and shallow lakes near Aveiro.

Sampling usually included plankton collection using a plankton net with 25–30 µm mesh size. When present, masses of filaments were also collected, as were often epiphytic organisms by squeezing submerged plants. A subsample was usually preserved in 4–6% Formalin and live samples were studied shortly after collection whenever possible.

Light micrographs were taken with a Zeiss Axioplan 2 imaging microscope equipped with a DP70 and a ColorView IIIu Olympus cameras. In a few cases, images were taken from still frames of videos recorded with a JVC digital, color video camera mounted on a Leitz Biomed light microscope.

Sample preparation for scanning electron microscopy (SEM) always followed the same protocol, except for the type of fixative. For field samples and cultures of armoured dinoflagellates, 5% Formalin, Lugol's solution or 25% ethanol were used. For cultured dinoflagellates with a delicate amphiesma fixation was made by adding 1 ml of culture to 1 ml of fixative mixture consisting of 1 part saturated $HgCl_2$ and 5 parts 2% OsO_4. Cells from all fixations were concentrated onto Isopore polycarbonate filters with 8-µm pore size, washed with distilled water and dehydrated through a graded ethanol series. The cells were critical-point-dried on the filters, which were then glued onto stubs with conductive, double-sided adhesive tape. The filters were sputter-coated with gold-palladium and were examined with a Hitachi S-4100 or with a Jeol JSM 5400 scanning electron microscope.

RESULTS

List of localities

This list contains the freshwater localities from which dinoflagellate species were reported in published works and those from our own records. Localities are grouped in

geographic units and numbered in sequence from north to south within each unit. As geographic units we use the Portuguese Provinces as defined by legislation of 1936. The Provinces were primarily founded on geographic criteria and their role in territorial administration was effectively ended by a 1959 decree; this makes their limits historically defined and therefore ideally stable for geographic reference points. Portuguese Provinces have been used in line with Spanish Provinces as species distribution units in Iberian floras, both for vascular plants (started in Castroviejo et al. 1986) and algae (Cirujano 2008). The limits of Portuguese Provinces and the approximate location of cited localities are given in Fig. 1. Each locality name is accompanied by its geographic coordinates based on the WGS84 datum.

Minho (Mi)

1 Salamonde reservoir; 41°41'31.82"N, 8° 5'19.27"W.

2 Venda Nova reservoir; 41°40'41.34"N, 7°58'50.22"W.

3 Ermal reservoir (Guilhofrei, Vieira do Minho); 41°35'9.66"N, 8°08'6.71"W.

Trás-os-Montes e Alto Douro (TM)

4 Lagoas do Marinho, Serra do Gerês; shallow ponds with *Sphagnum* at alt. 1150 m; 41°45'45.41"N, 8°02'41.10"W.

5 Alto Rabagão reservoir; 41°44'25.74"N, 7°51'18.82"W.

6 Carvalhelhos (Boticas), puddle (leg. Henrique Caetano); 41°41'23"N, 7°43'49"W.

7 Azibo reservoir; 41°33'31.63"N, 6°53'22.58"W.

Beira Alta (BA)

8 Vale do Rossim reservoir (Serra da Estrela); 40°23'55.87"N, 7°35'10.35"W.

9 Outlet of Vale do Rossim reservoir; 40°23'59.92"N, 7°35'22.22"W.

10 Covão da Malhada reservoir (Serra da Estrela); 40°23'28.64"N, 7°36'1.87"W.

11 Covão do Curral reservoir (Covão do Vidual, Serra da Estrela); 40°22'16.91"N, 7°38'29.29"W.

12 Lagoa Comprida reservoir (Serra da Estrela); 40°21'43.43"N, 7°38'49.23"W.

13 Puddles near Lagoa Comprida; 40°21'40.23"N, 7°39'8.45"W.

14 Lagoa Escura (Serra da Estrela); 40°21'19.48"N, 7°38'12.90"W.

15 Small reservoir near Torre (Serra da Estrela); 40°19'35.22"N, 7°36'30.49"W.

16 Vale de Espinho (River Côa); 40°16'55.89"N, 6°55'54.93"W.

Beira Baixa (BB)

17 Santa Luzia reservoir; 40°05'27.44"N, 7°51'24.84"W.

18 Cabril reservoir; 39°55'12.33"N, 8°07'51.45"W.

19 Idanha reservoir (formerly Marechal Carmona); 39°56'46.39"N, 7°11'57.49"W.

20 Pracana reservoir; 39°33'54.51"N, 7°48'35.26"W.

Beira Litoral (BL)

21 Temporary puddle in Cacia, Aveiro; approximately 40°40'46"N, 8°35'33"W.

22 River Vouga, ditch at Taboeira, Aveiro; 40°39'27.66"N, 8°34'26.34"W.

23 Pool in park in Aveiro (Baixa de Santo António); 40°38'19.19"N, 8°39'21.98"W.

24 Pond in park in Aveiro (Infante D. Pedro park); 40°38'7.23"N, 8°39'12.69"W.

25 Waste water lagooning tank in the University Campus, Aveiro; 40°38'7.14"N, 8°39'32.03"W.

26 Clean water tank in the University Campus, Aveiro; 40°38'4.52"N, 8°39'30.21"W.

27 Puddle formed by revolved earth in the University Campus, Aveiro (no longer exists); 40°38'4.29"N, 8°39'34.97"W.

28 Water-covered sediment on sidewalk, University Campus, Aveiro; 40°38'1.75"N, 8°39'30.56"W.

29 River Vouga, ditch at Parque da Balsa, Eixo, Aveiro; 40°37'50.90"N, 8°33'33.30"W.

30 River Vouga, ditch at Segadães, Aveiro; 40°36'31.58"N, 8°30'59.79"W.

31 Ponds in farm at Gafanha da Boavista, near Vista Alegre, Ílhavo – Four medium size ponds (about 150–250 m in diameter) used for fishing and water supply; pond I: 40°35'44.70"N , 8°41'49.66"W; pond II: 40°35'44.41"N, 8°41'58.89"W; pond III: 40°36'13.54"N, 8°41'49.17"W; pond IV: 40°36'20.11"N, 8°41'43.29"W.

32 Pateira de Fermentelos; 40°34'26.52"N, 8°30'49.13"W.

33 Flooded area in Ribeiro do Pano stream, Mamodeiro, Aveiro; 40°33'50.28"N, 8°33'49.94"W.

34 Flooded area in Ribeiro da Palha stream, Nariz, Aveiro; 40°33'14.42"N, 8°34'5.73"W.

35 Ponds in clay extraction sites, abandoned Ceramics factory, Nariz, Aveiro; 40°32'42.25"N, 8°35'25.33"W.

36 Lagoa de Calvão; 40°28'35.23"N, 8°42'05.76"W.

37 Barrinha de Mira; 40°26'57.81"N, 8°47'50.14"W.

38 Lagoa de Mira; 40°26'29.67"N, 8°45'20.96"W.

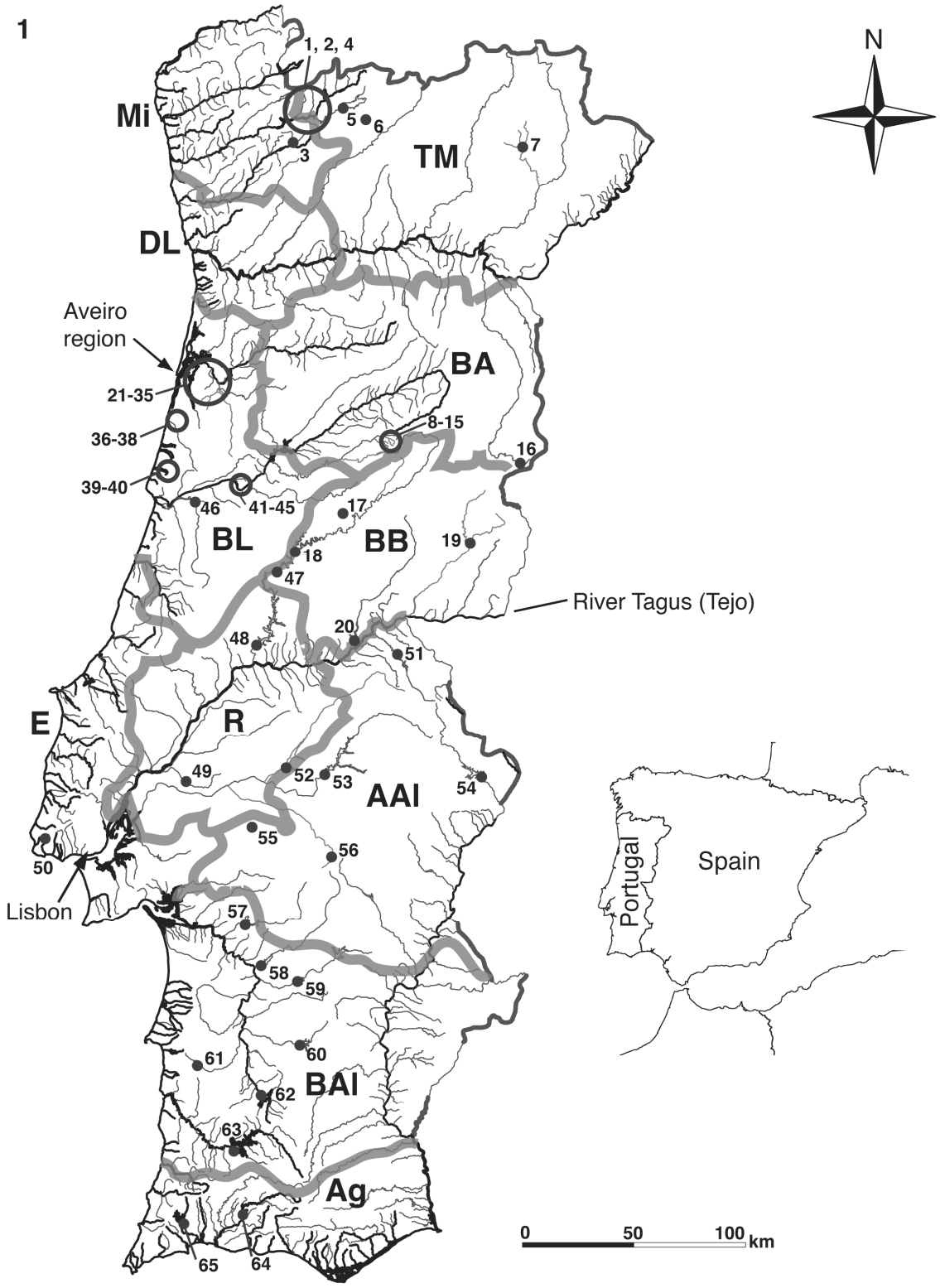


Fig. 1. Map of Portugal showing the main river network and the boundaries of Provinces (from north to south): Mi, Minho; TM, Trás-os-Montes e Alto Douro; DL, Douro Litoral; BA, Beira Alta; BL, Beira Litoral; BB, Beira Baixa; R, Ribatejo; E, Estremadura; AAl, Alto Alentejo; BAl, Baixo Alentejo; Ag, Algarve. Numbers indicate localities from which dinoflagellates are reported, both from published works and from our collections. See list of localities in text.

39 Lagoa da Vela; 40°16'9.70"N, 8°47'30.66"W.

40 Lagoa das Braças; 40°14'36.35"N, 8°48'17.23"W.

41 River Mondego at Coimbra; 40°12'51.39"N, 8°26'19.69"W.

42 River Mondego, Poço do Almegue, Coimbra. Samples from this location were taken in 1958, in an area very close to where now stands the Coimbra dam (see coordinates of locality 41).

43 Botanic Garden in Coimbra; 40°12'20.87"N, 8°25'17.07"W.

44 Puddles near Coimbra (precise location not given).

45 River Ceira, just before the confluence with River Mondego, Coimbra; 40°10'58.22"N, 8°23'44.64"W.

46 Madriz Marsh; 40°07'38.19"N, 8°38'24.20"W.

47 Bouçã reservoir; 39°51'5.13"N, 8°13'1.54"W.

Ribatejo (R)

48 Castelo do Bode reservoir (River Zêzere); 39°32'36.61"N, 8°19'1.15"W.

49 Magos reservoir; 38°59'37.06"N, 8°41'16.14"W.

Estremadura (E)

50 Mula reservoir; 38°45'54.81"N, 9°25'21.93"W.

Alto Alentejo (AAl)

51 Poio reservoir; 39°30'57.68"N, 7°34'53.07"W.

52 Montargil reservoir; 39°03'19.38"N, 8°10'28.81"W.

53 Maranhão reservoir; 39°00'50.95"N, 7°58'26.89"W.

54 Caia reservoir; 39°00'02.72"N, 7°08'50.79"W.

55 Puddle near the road Lavre-Ciborro, Montemor-o-Novo; 38°47'46.75"N, 8°20'27.14"W.

56 Divor reservoir; 38°41'52.45"N, 7°55'32.16"W.

Baixo Alentejo (BAl)

57 Pego do Altar reservoir (formerly Salazar); 38°25'4.80"N, 8°23'22.63"W.

58 Vale do Gaio reservoir (Trigo de Morais); 38°14'55.61"N, 8°17'35.45"W.

59 Odivelas reservoir; 38°11'9.72"N, 8° 6'47.41"W.

60 Roxo reservoir; 37°55'48.26"N, 8°04'42.25"W.

61 Campilhas reservoir; 37°50'38.40"N, 8°37'21.88"W.

62 Monte da Rocha reservoir; 37°43'28.62"N, 8°17'15.44"W.

63 Santa Clara reservoir; 37°30'55.62"N, 8°26'25.40"W.

Algarve (Ag)

64 Arade reservoir; 37°14'29.06"N, 8°22'29.30"W.

65 Bravura reservoir; 37°12'11.69"N, 8°41'58.28"W.

List of species

Dinoflagellate taxonomy at genus, family and even order level has been substantially modified during the last 20 years and many areas of uncertainty remain. Adopting the recently proposed genera that we think are well-founded together with the higher-level classification of the latest freshwater dinoflagellate floras would be awkward and confusing. However, introducing a classification unfamiliar to most users, even if it were more firmly established than we are able to make it with the present knowledge, would hinder rather than help finding individual species in the checklist. Therefore, species are given in alphabetic order with the following format: the species entry contains the name of the species in boldface and its author or authors in standard form; then are given, in separate lines, homotypic synonyms, starting with the basionym, if there is one; heterotypic synonyms cited for Portugal, if any exist, are listed next, followed where appropriate by a list of names that, in our opinion, have been misapplied and belong in this entry; in the next line is the list of localities from which the species has been recorded, given by their code numbers and grouped by Province, with localities not previously published marked with an asterisk (except for taxa cited for the first time, for which all localities are, of course, new). Published reports of the species are referred in chronological order after the name that they actually cited; previously unreported taxa are marked as new records for the region.

Trying to form an opinion about the identity of cited taxa is dependent upon any indications that the original authors may have given about the specimens examined. In the majority of cases nothing was given that may assist taxonomical decisions; most helpful are the few instances in which illustrations were provided and these have been indicated in the references. Most entries end with a brief note addressing issues that are deemed appropriate, as recent taxonomic changes, the basis for heterotypic synonymies or information on times of the year when we recorded particular taxa.

Readers searching for particular species are requested to take into account recent changes in generic assignment. If a species name, as used in recent floras (Starmach 1974, Popovský & Pfiester 1990) does not appear in its alphabetic position use the following guideline: for species of *Amphidinium* see in *Prosoaulax*; for *Bernardinium* see *Esoptrodinium*; for *Gymnodinium* see *Biecheleria*, *Gyrodinium*, *Jadwigia* or *Woloszynskia*; for *Katodinium* see *Opisthoaulax*; for *Peridiniopsis* see *Durinskia* or *Tyrannodinium*; for *Peridinium* see *Chimonodinium*, *Durinskia*, *Glochidinium*, *Palatinus* or *Parvodinium*; for *Woloszynskia* see *Biecheleria* or *Jadwigia*.

Biecheleria pseudopalustris (J.Schiller) Moestrup, K.Lindberg et Daugbjerg

BASIONYM: *Gymnodinium pseudopalustre* J.Schiller 1932: 400, fig. 418.

Biecheleria pseudopalustris (J.Schiller) Moestrup, K.Lindberg et Daugbjerg 2009: 213.

HETEROTYPIC SYNONYM: *Gymnodinium excavatum* Nygaard 1945: 52, text-fig. 20. – Oliveira (1982b).

LOCALITIES: **BB**: *20*.

This species is known as *Woloszynskia pseudopalustris* (J.Schiller) Kisselev *ex* Elbrächter (Kremp et al. 2005: 635) in recent floras (Starmach 1974, Popovský & Pfiester 1990). Its eyespot, made of layered vesicles with crystal-like contents, combined with a particular apical elongated vesicle and a spherical cyst type covered with thin spines led to its separation into a new genus, *Biecheleria* Moestrup, K.Lindberg et Daugbjerg (2009: 213). Large subunit rDNA-based phylogenies support its affinity with the Suessiales (Moestrup et al. 2009). Stosch (1973) demonstrated that *G. excavatum* is part of the life cycle of this species and we have verified that cultures initiated from vegetative cells with *G. excavatum* morphology developed into planozygotes with the morphology originally ascribed to *G. pseudopalustre* and ultimately formed the typical cysts of the species (Calado & Craveiro, unpublished).

Borghiella dodgei Moestrup, Gert Hansen et Daugbjerg Figs 2–8

Borghiella dodgei Moestrup, Gert Hansen et Daugbjerg 2008: 57, figs 1–5, 7–11. – New record for the region.

LOCALITIES: **BL**: *34*, *35*.

The genus *Borghiella* Moestrup, Gert Hansen et Daugbjerg was established for a group of woloszynskioid species with an eyespot made of intraplastidial lipid globules and an extraplastidial vesicle containing brick-like units, two parallel, elongated vesicles in the cell apex (one with a row of knobs), and smooth cysts (Moestrup et al. 2008). Species of *Borghiella* are rather difficult to identify in the light microscope and the identity of our material was established through a combination of light and scanning electron microscopy of cultures established from cells isolated in November 2010. Although the epicone of cells in our cultured strains, as shown in Figs 5 and 6, is somewhat more rounded (i.e., less conical) than the ones shown in Moestrup et al. (2008) the limits of morphological variability of species of *Borghiella* are largely unknown and we therefore refer our material to *B. dodgei*.

Ceratium cornutum (Ehrenberg) Claparède et J.Lachmann

BASIONYM: *Peridinium cornutum* Ehrenberg 1832: 75.

Ceratium cornutum (Ehrenberg) Claparède et J.Lachmann 1859: 394. – Calado & Craveiro (1993).

LOCALITIES: **BL**: *32*, *33**.

We recorded *C. cornutum* several times in the autumn and once in mid-June.

Ceratium furcoides (Levander) Langhans Figs 9–10

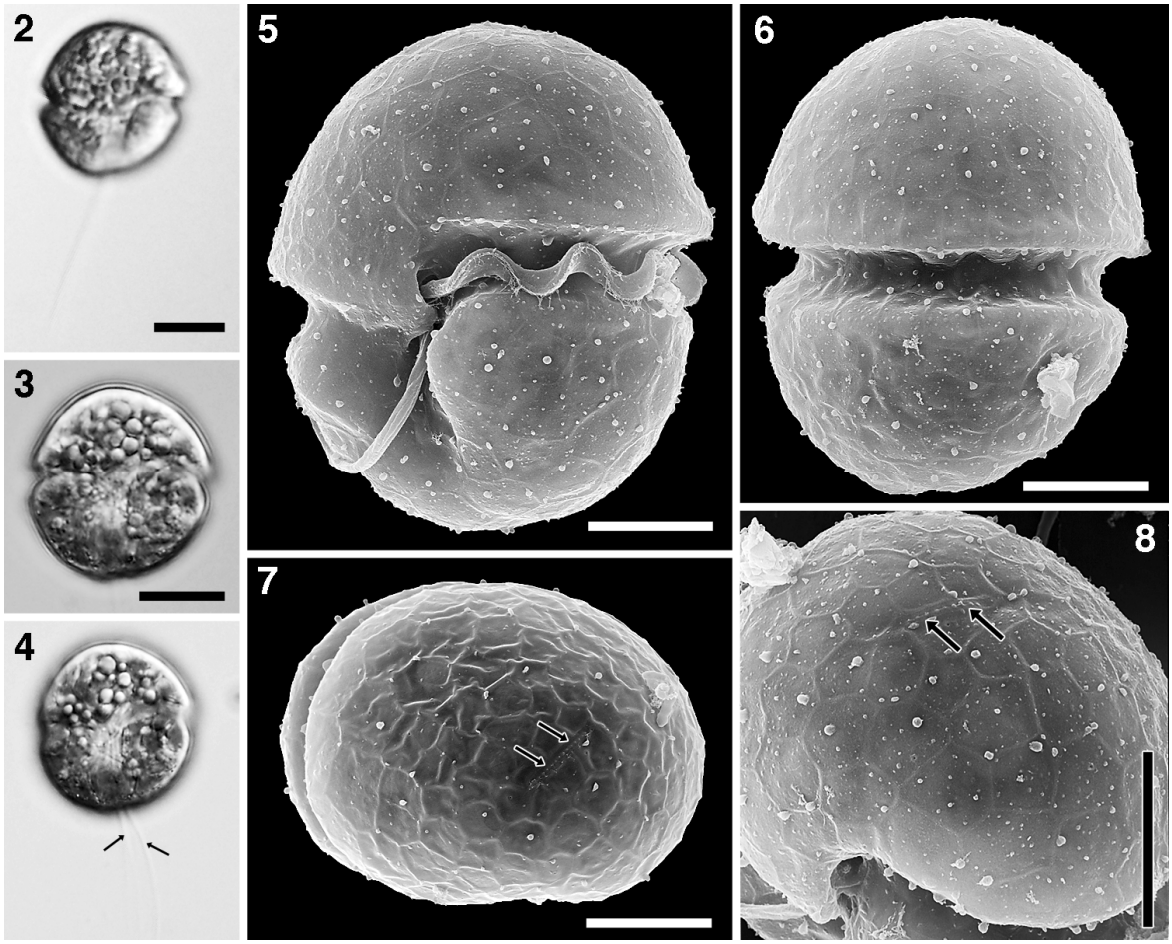
BASIONYM: *Ceratium hirundinella* var. *furcoides* Levander 1894: 53, pl. II, fig. 24.

Ceratium furcoides (Levander) Langhans 1925: 602. – Craveiro & Santos (1999: pl. I, fig. 19), Santos et al. (2002: pl. 4, figs 18, 19).

MISAPPLIED NAME: *Ceratium hirundinella*. – Frade (1954: figs 6, 7; 1957: figs 3, 4), Nauwerck (1962, as *"C. hirundinella* f. *furcoides"*, one of the morphotypes ascribed to *C. furcoides*).

LOCALITIES: **BA**: *11*; **BL**: *25**, *30**, *31**, *32**, *33**, *35**, *37**, *38**, *39*, *40**, *41*; **R**: *48*.

This is by far the most common species of *Ceratium* that we have found in freshwater, both in recent and in older, fixed samples. The figures given by Frade (1954, 1957) show the characteristic concave shape of the epithecal base and we refer them to this species. We recorded the species during all seasons but the most abundant populations occurred regularly in the autumn.



Figs 2–8. *Borghiella dodgei* from cultures started by isolation of cells from Ribeiro da Palha (*34*) in November 2010. Figs 2–4. LM of cells in ventral view. Both scale bars = 10 µm. Figs 2–3. Typical cell shape is shown. Fig. 4. Cell slightly squashed, with two longitudinal flagella (arrows) indicating a planozygote. Same scale as Fig. 2. Figs 5–8. SEM of cells in different views showing the amphiesma formed by small hexagonal vesicles. All scale bars = 5 µm. Fig. 5. Ventral view with longitudinal and transverse flagella and descending cingulum. Fig. 6. Dorsal view. Fig. 7. Apical view with the ventral side toward the bottom of the picture, showing an apical pair of elongated amphiesmal vesicles (arrows). Fig. 8. Higher magnification of apex showing a row of knobs (arrows) in one of the apical, elongated vesicles.

Ceratium hirundinella (O.F.Müller) Dujardin Figs 11–13

BASIONYM: *Bursaria hirundinella* O.F.Müller 1773: 63.

Ceratium hirundinella (O.F.Müller) Dujardin 1841: 377. – Nauwerck (1962, as *"C. hirundinella* f. *carinthiacum"*, one of the morphotypes ascribed to *C. hirundinella*), Moita (1976), Oliveira (1982a, 1982b, 1982c), Oliveira (1984a, 1984c), Ferreira (1987), Andrade et al. (1988), Coutinho (1990), Calado (1993), Calado & Craveiro (1993), Santos et al. (2002).

LOCALITIES: **Mi**: *2*; **TM**: *5*; **BA**: pond near *12*; **BB**: *17*, *18*, *19*, *20*; **BL**: *37*, *38*, *39*, *40*; **R**: *48*, *49*; **E**: *50*; **AAl**: *52*, *53*, *54*, *56*; **BAl**: *57*, *59*, *60*, *61*, *63*; **Ag**: *64*, *65*.

Ceratium hirundinella is a very characteristic, eye-catching species, and it is not surprising that it is the most cited dinoflagellate name in Portugal. However, before Popovský & Pfiester (1990), mainly based on Hickel (1988a), recognized *C. furcoides* as a different species most authors would apply the name *C. hirundinella* to any freshwater *Ceratium* with a straight anterior and two or three posterior horns. A correlation between the typical bell-shaped base of the epitheca of *C. hirundinella* with the presence of four apical plates that reach the apex, contrasting with the presence of a short apical plate 4 in the more concave *C. furcoides*, has been demonstrated (Calado & Larsen 1997). However, shape variation in these species is extensive and it is not always straightforward to deduce the length of apical plate 4 from the format of the epitheca. Figures 11–13 demonstrate *C. hirundinella* from samples fixed 24 years ago. The remaining records of *C. hirundinella*, all undocumented, are doubtful.

Ceratium rhomvoides B.Hickel Figs 14–18

Ceratium rhomvoides B.Hickel 1988b: 49, figs 1–18. – New record for the region.

LOCALITIES: **BL**: *31*.

We recorded cells with the features shown in Figs 14–18 in October 2010 and in November 2011. The distinctly shorter appearance than is usual in *C. furcoides* (Figs 9, 10) and the more deeply reticulated plate surface correspond to Hickel's (1988b) description of *C. rhomvoides*. This and other variations around the *C. furcoides–hirundinella* theme, including life cycle modifications of these species (e.g., gametes), need to be examined further to establish species limits in this group.

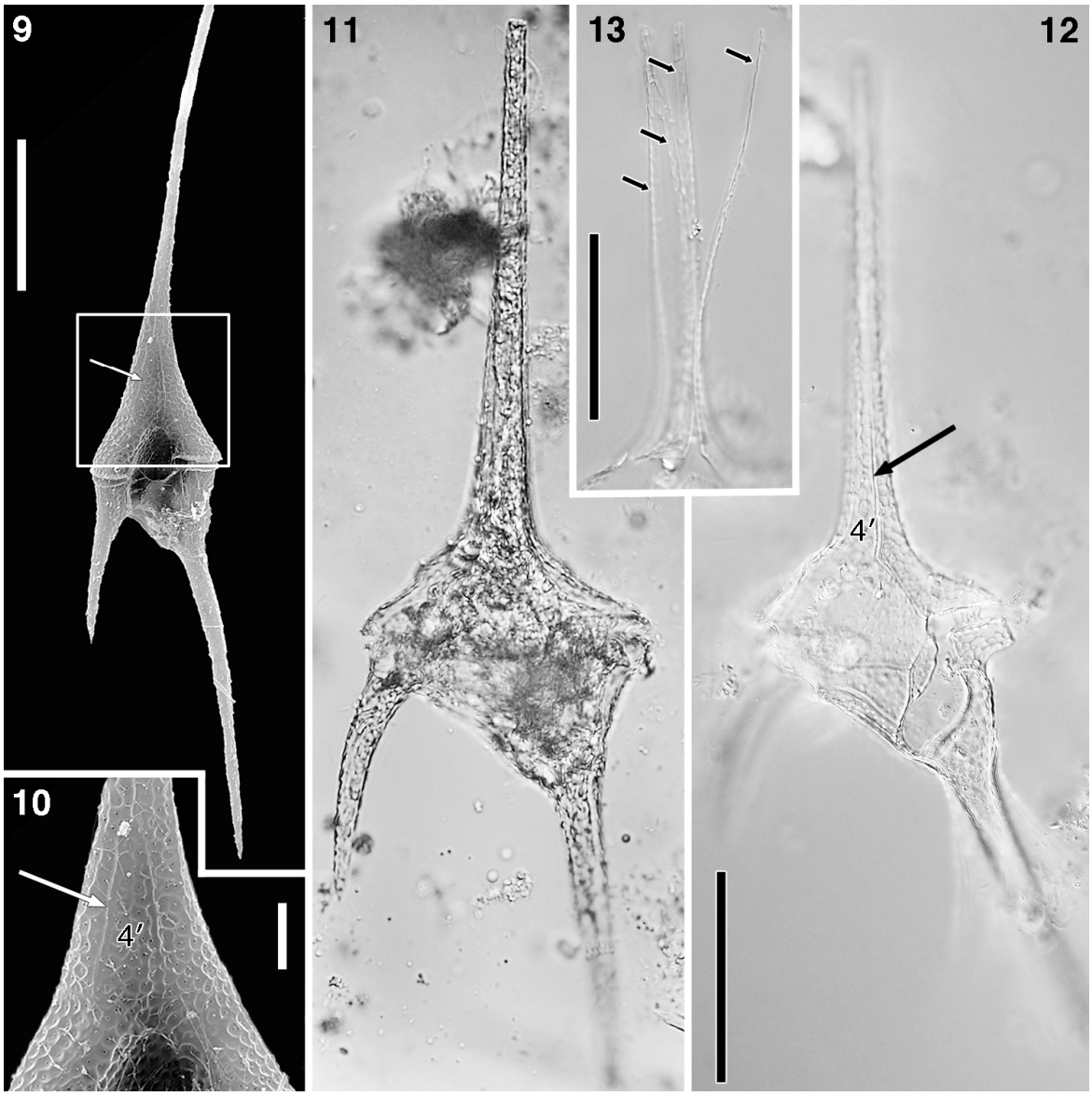
Chimonodinium lomnickii (Wołoszyńska) Craveiro, Calado, Daugbjerg, Gert Hansen et Moestrup Figs 19–20

BASIONYM: *Peridinium lomnickii* Wołoszyńska 1916: 267, pl. 10, figs 25–29.

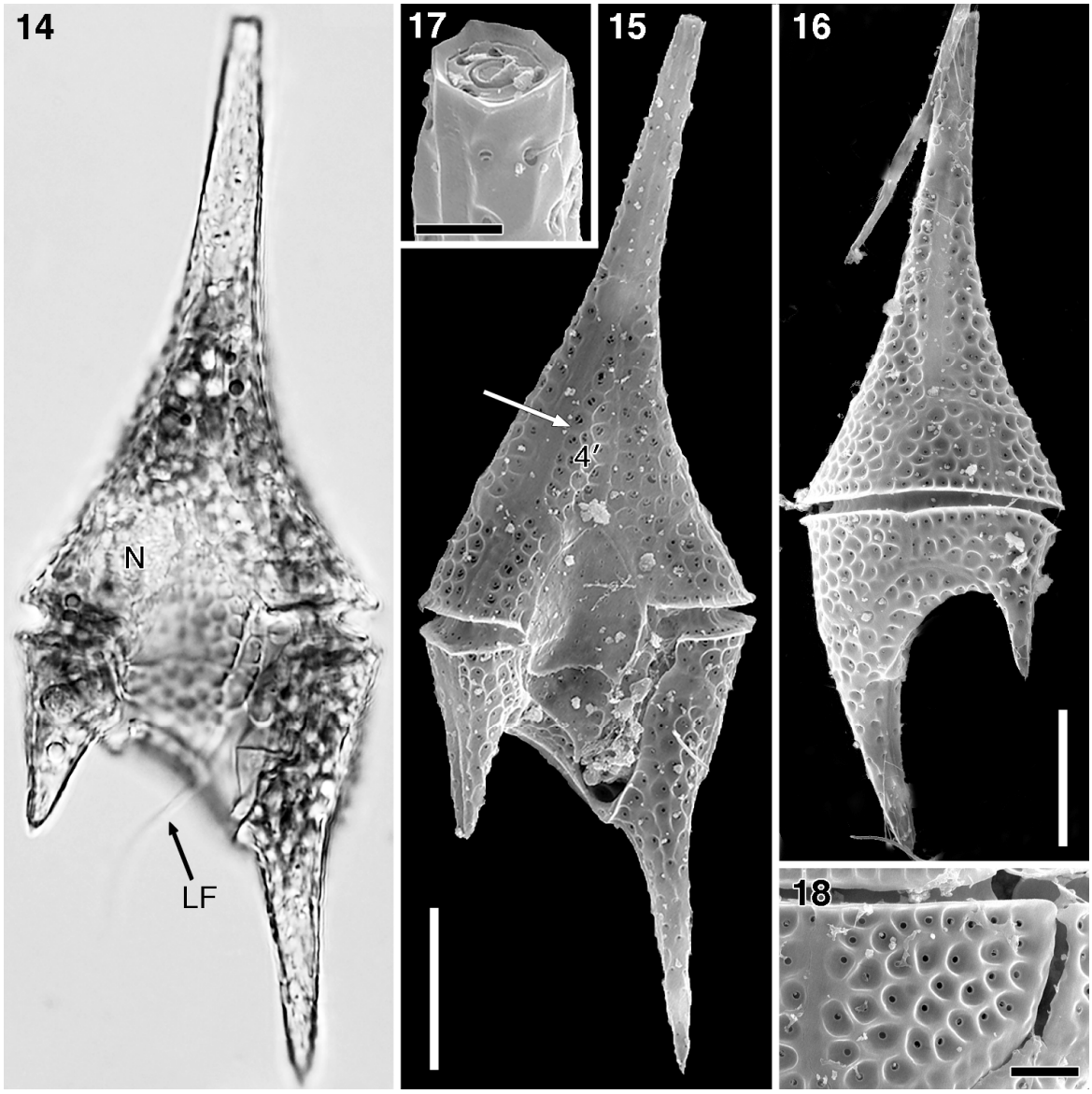
Chimonodinium lomnickii (Wołoszyńska) Craveiro, Calado, Daugbjerg, Gert Hansen et Moestrup 2011: 605, 606. – Craveiro et al. (2011).

LOCALITIES: **BA**: *11**; **BL**: *29**, *30**, *32*, *33**, *37**.

The tabulation of this species differs from that of *Peridinium* sensu stricto in the presence of an apical pore complex and six cingular plates. The cells lack pyrenoids and were shown to have a small peduncle supported by 4–5 overlapping rows of microtubules,



Figs 9–13. *Ceratium furcoides* and *C. hirundinella*. Figs 9–10. SEM of *C. furcoides* from a pond in Gafanha da Boavista (*31*), collected in October 2010. Fig. 9. Ventral view of complete cell with plate 4′ short (white arrow). Fig. 10. Higher magnification showing plate 4′ (white arrow) and ornamentation. Figs 11–13. LM of *C. hirundinella* collected in Lagoa da Vela (*39*) in August 1988. Figs 11–12. Ventral view showing the typical cell shape and the long plate 4′ (black arrow). Scale bar applies to both Figs. Fig. 6. Squashed cell showing the 4 long apical plates (arrows) that form the apical horn. Scale bars = 50 µm in all Figs except for Fig. 10, in which it represents 10 µm.



Figs 14–18. *Ceratium rhomvoides* from a pond in Gafanha da Boavista (*31*) sampled in October 2010. Fig. 14. Ventral view of a live cell in LM. LF, longitudinal flagellum; N, nucleus. Same scale as Fig. 15. Figs 15–16. Ventral and dorsal views, SEM. Short plate 4′ (white arrow) in ventral view. Scale bars = 20 µm. Fig. 17. Detail of the apical pore region, SEM. Scale bar = 2 µm. Fig. 18. Higher magnification of a post-cingular plate showing deep reticulated ornamentation. Scale bar = 5 µm.

and a pusular system with well-defined pusular tubes; these features, and the production of non-calcareous cysts, set it appart from typical *Scrippsiella* species (which have the same general tabulation) and led to its transfer to the new genus *Chimonodinium* Craveiro, Calado, Daugbjerg, Gert Hansen et Moestrup (Craveiro et al. 2011). Partial large subunit rDNA-based phylogenetic hypotheses also suggest a closer relationship with the

pfiesteriaceans then with *Scrippsiella* (Craveiro et al. 2011). It was found in winter and early spring in the coastal area and in Serra da Estrela at 1500 m in April.

Cystodinedria inermis (Geitler) Pascher Figs 21–24

BASIONYM: *Raciborskia inermis* Geitler 1943: 173, figs 2e–m, 3d–m, 4a–e.

Cystodinedria inermis (Geitler) Pascher 1944: 381. – New record for the region.

LOCALITIES: **BA**: *9*.

Recorded only in April–May 2011 at 1425-m altitude, attached to filaments of *Mougeotia* and *Tribonema*. The cells had yellowish chloroplasts, radially disposed in some specimens, apparently parietal in others. An eyespot was visible in most cells, in the face next to the substrate filament, but there were no furrows in the cytoplasm of the attached cells. The filaments were not visibly altered at the place of attachment of the dinoflagellates.

Cystodinium cornifax (A.J.Schilling) G.A.Klebs

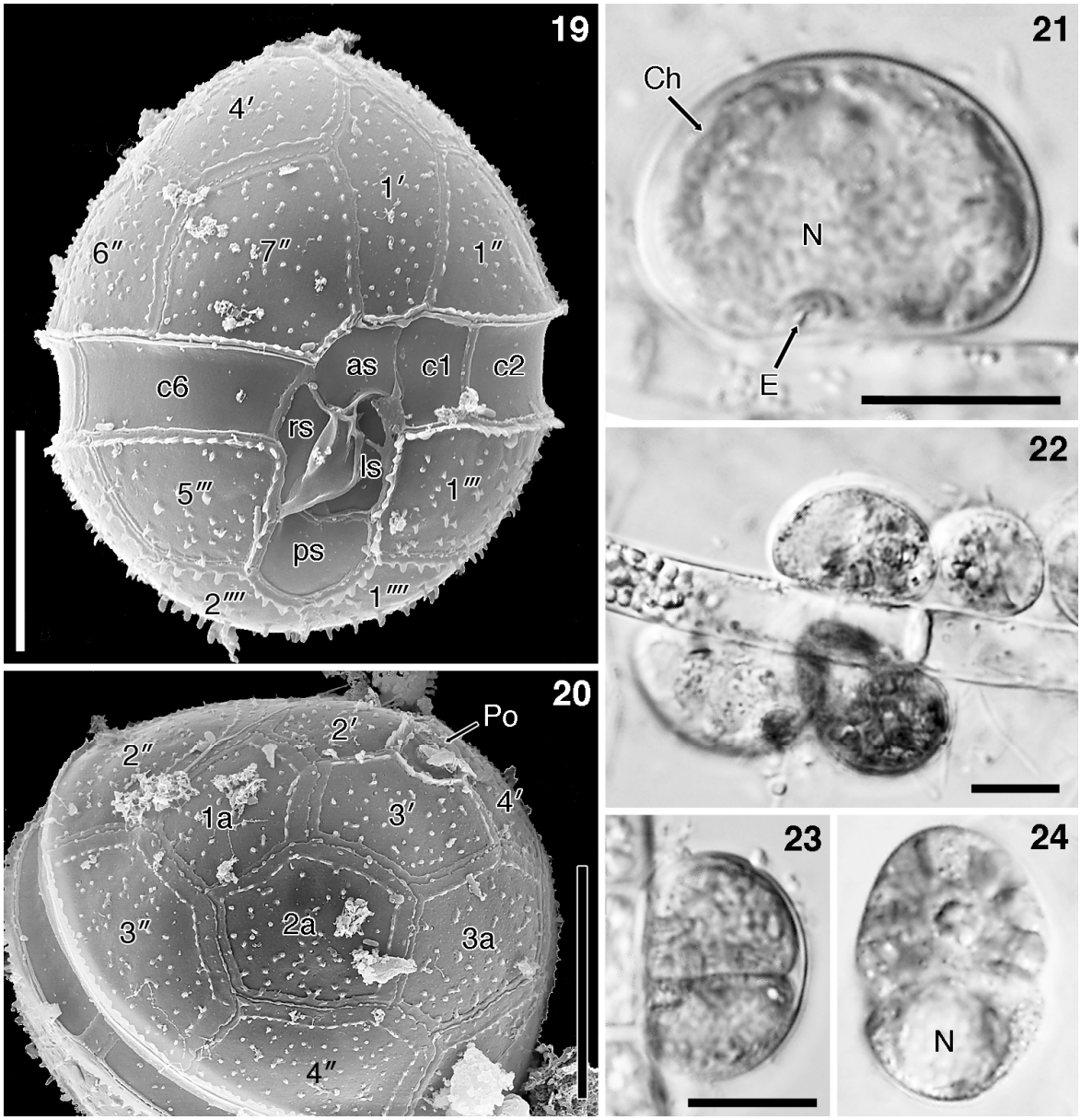
BASIONYM: *Glenodinium cornifax* A.J.Schilling 1891a: 285, pl. VIII, fig. 1, pl. X, figs 1–5, 18.

Cystodinium cornifax (A.J.Schilling) G.A.Klebs 1912: 384, 442.

HETEROTYPIC SYNONYM: *Cystodinium steinii* G.A.Klebs 1912: 381, 442, text-fig. 3, pl. X, fig. 2. – Rodrigues (1961: pl. I, fig. 3).

LOCALITIES: **BL**: *21*.

Although the treatment of *Cystodinium* in Popovský & Pfiester (1990) suffers, in our opinion, form excessive lumping, we follow their opinion that *C. steinii* is conspecific with *C. cornifax* because the main characters used for separating these two entities, namely a noticeable difference in length between the two terminal spines (Matvienko & Litvinenko 1977), or a difference in spine thickness (Starmach 1974) are not clear in the original descriptions and seem to underestimate intraspecific variability.



Figs 19–24. *Chimonodinium lomnickii* and *Cystodinedria inermis*. Figs 19–20. SEM of *Chimonodinium lomnickii* from Pateira de Fermentelos (*32*), January 2006. Fig. 19. Ventral view with tabulation marked in Kofoidean notation. The cell swelled slightly under the electron beam, and clearly shows the furrow plates. Fig. 20. Dorsal-apical view. Note the three anterior intercalary plates (1a, 2a and 3a) and the apical pore (Po) typical of this species. Figs 21–24. LM of live cells of *Cystodinedria inermis* from an outlet of Vale do Rossim reservoir (*9*), April 2011. Fig. 21. Immotile cell attached to a filament of *Mougeotia* sp. Note the large central nucleus (N), the chloroplast lobes (Ch) on the surface and the eyespot (E). Fig. 22. Group of cells attached to a filament of *Mougeotia* sp. Fig. 23. Dividing cell attached to a filament of *Tribonema* sp. Fig. 24. Motile cell with nucleus on the hypocone. Same scale as Fig. 23. All scale bars = 10 µm.

Dinococcus oedogonii (P.G.Richter) Fott

BASIONYM: *Rhizophydium oedogonii* P.G.Richter 1897: 12, fig. 6.

Dinococcus oedogonii (P.G.Richter) Fott 1960: 149.

HETEROTYPIC SYNONYMS: *Cystodinium brevipes* Geitler 1928, *pro parte*: p. 68, 81, text-fig. 1a, 1d, pl. 7, fig. 1. – Santos (1976: pl. V, fig. 2; pl. XVII, figs 3, 4).

Raciborskia bicornis Wołoszyńska 1919: 199, pl. 14, figs 15–17. – Rino (1967: pl. IV, figs 1, 2).

LOCALITIES: **BA**: *13*; **BL**: *43*.

The synonymy follows Geitler (1943).

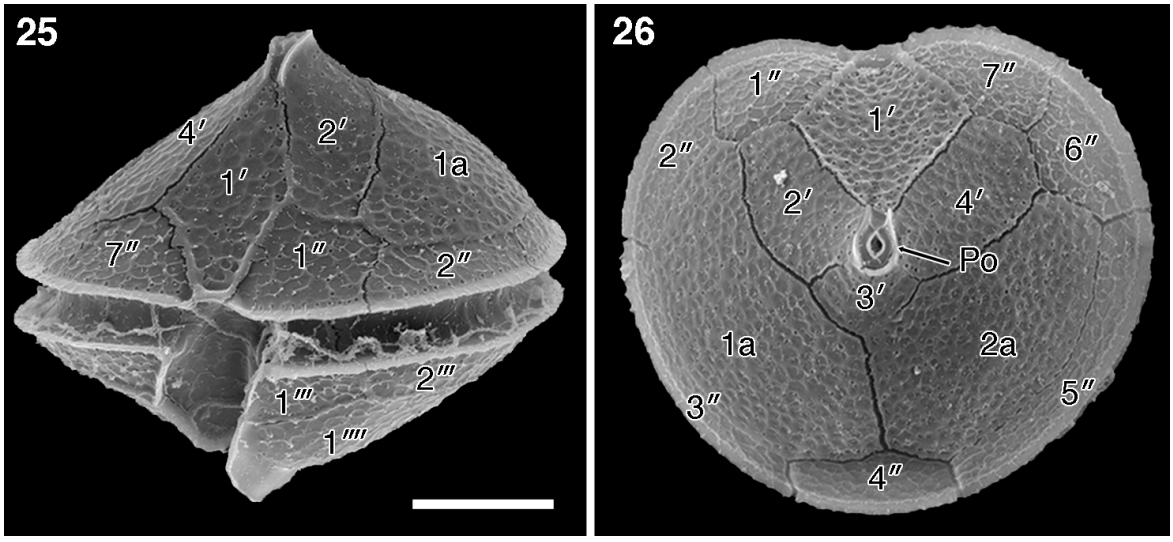
Diplopsalis acuta (Apstein) Entz Figs 25–26

BASIONYM: *Glenodinium acutum* Apstein 1896: 152, fig. 54.

Diplopsalis acuta (Apstein) Entz 1904: 12. – New record for the region.

LOCALITIES: **BL**: *31*.

Although this heterotrophic species is classified in the genus *Diplopsalis* Bergh in several freshwater floras (e.g., Huber-Pestalozzi 1950, Popovský & Pfiester 1990), Lebour considered it to represent a different genus and named it *Entzia acuta* (Apstein) M.Lebour (1922: 808). On the basis of Sebestyén's (1935) observations indicating that the previously described genus *Kolkwitziella* Er.Lindemann represents the cyst stage of *D. acuta* the combination *Kolkwitziella acuta* (Apstein) Elbrächter (1993: 174) was established. Comparison with the type species of *Diplopsalis*, the marine *D. lenticula* Bergh, by modern methods is needed to determine its phylogenetic affinities. The species was found regularly in the ponds of Gafanha da Boavista where it was sometimes frequent, especially in the autumn.



Figs 25–26. SEM of *Diplopsalis acuta* collected from a pond in Gafanha da Boavista (*31*) in November 2010. Fig. 25. Ventral view showing tabulation (Kofoidean notation). Scale bar = 10 µm. Fig. 26. Apical view. Po, apical pore. Same scale as Fig. 25.

Durinskia baltica (Levander) Carty et El.R.Cox Figs 27–31

BASIONYM: *Glenodinium balticum* Levander 1894: 52.

Durinskia baltica (Levander) Carty et El.R.Cox 1986: 200. – New record for the region.

LOCALITIES: **BL**: *22*, *23*, *24*, *31*, *32*, *33*, *34*, *38*.

Durinskia belongs to a group of phylogenetically related dinoflagellates that have derived their plastids from a diatom endosymbiont (Takano et al. 2008). As is common in this group, our specimens are dikaryotic (Fig. 28). The species has been reported from a large range of salinities (Carty & Cox 1986); all the occurrences given here are from freshwater localities and in all cases general morphology, tabulation and the appearance of cell contents were consistent with Figs 27–31. We recorded the species during all seasons of the year.

Esoptrodinium gemma Javornický

Esoptrodinium gemma Javornický 1997: 36, fig. 2, pl. ("Table") II .– Calado et al. (2006).

LOCALITIES: **BL**: *28*.

The genus *Esoptrodinium* Javornický (1997: 35) was proposed for dinoflagellates with a left-hand orientation of the transverse flagellum and an astonishing morphological resemblance to *Bernardinium* Chodat (1924: 40). The difference between the type species

of the two genera, *E. gemma* and *B. bernardinense* Chodat (1924: 41, fig. VII), is the right-hand, mirror-symmetrical orientation of the transverse flagellum of *Bernardinium* as originally depicted by Chodat and redescribed by Javornický (1997). The possibility of mistakes being at the origin of the interpretation that the orientation of *Bernardinium* is the reverse of what is usual in dinoflagellates was discussed by Calado et al. (2006). Although we list the name that was use to report the species for Portugal, which unambiguously indicates the left-hand orientation of its transverse flagellum, we acknowledge the possibility that the two names are synonyms, in which case the species should be called *B. bernardinense*.

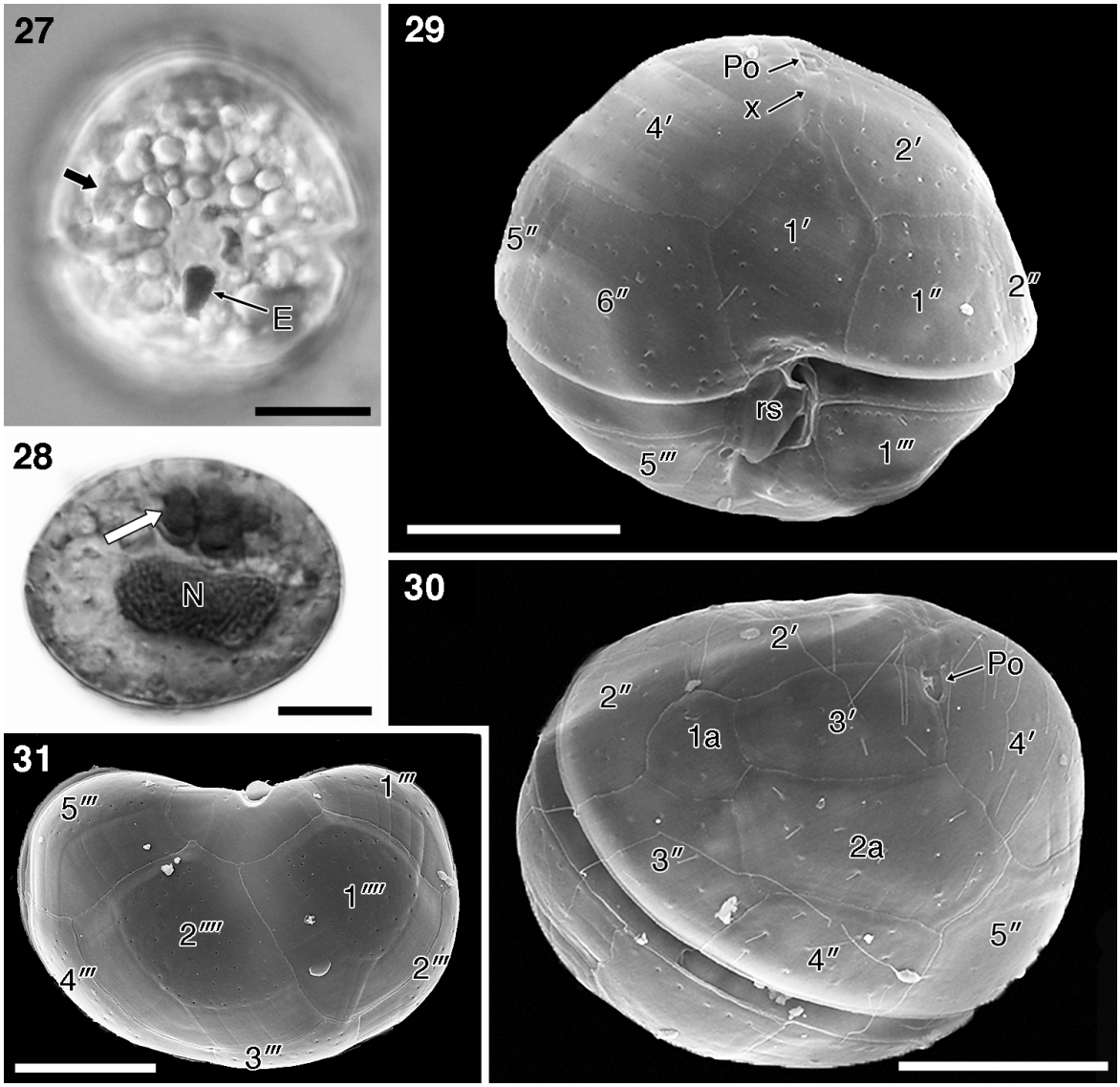
Glochidinium penardiforme (Er.Lindemann) Boltovskoy

BASIONYM: *Peridinium penardiforme* Er.Lindemann *in* Schröder 1919: 654, fig. 1 (see also Lindemann 1920: 126, figs 10–15). – Oliveira (1984a).

Glochidinium penardiforme (Er.Lindemann) Boltovskoy 1999: 99.

LOCALITIES: **BL**: *31**, *32**, *35**; **BAL**: *59*.

This species is named *Peridiniopsis penardiformis* (Er.Lindemann) Bourrelly (1968: 8) in recent floras (Starmach 1974, Popovský & Pfiester 1990). The genus *Glochidinium* Boltovskoy (1999: 98) was separated from *Peridinium* and *Peridiniopsis* on the basis of tabulation differences, especially its unusual cingulum with only three plates. Its phylogenetic position within the peridinioids needs to be established by modern methods. The species is somewhat enigmatic in having been reported both with and without chloroplasts (Lefèvre 1932, Starmach 1974). Our Portuguese records of the species are based on specimens with few cytoplasmic yellowish bodies and no clear chloroplasts, except for a large population (about 200 cells/ml) found in Pateira de Fermentelos in June 1989, in which all cells looked as full of chloroplast lobes as does a common peridinin-containing species such as *P. cinctum*. In addition, we have examined populations from Danish ponds totally composed of cells with fully colourless contents, although otherwise their morphology and tabulation completely matched the one described for the species. The nutritional strategies and the genetic diversity of these organisms are obviously in need of attention.



Figs 27–31. *Durinskia baltica* from culture. Fig. 27. LM of living cell from a culture started by isolation of cells from Lagoa de Mira (*38*) in November 2010. E, eyespot; thick arrow, chloroplast lobes. Fig. 28. LM of a cell stained with acetocarmine showing the typical nucleus of the dinoflagellate (N) and the nucleus of the endosymbiont (white arrow). Culture started by isolation of cells from a pond in a park in Aveiro (*24*) in March 2011. Figs 29–31. Ventral, dorsal-apical and antapical views of cells with typical tabulation, SEM. Po, apical pore; x, canal plate. Note the typical intercalary apical plates (1a and 2a) with a large difference in size. Cells from the same culture as Fig. 28. All scale bars = 10 µm.

Gloeodinium montanum G.A.Klebs

Gloeodinium montanum G.A.Klebs 1912: 414, 445, text-fig. 13, pl. X, fig. 5. – Rino (1969: pl. III, fig. 1).

LOCALITIES: **TM**: *4**; **BA**: *13*, *16**.

Both Rino's (1969) report and our own records are from mountain areas, above 800 m, in spring and summer. Only the immobile, so-called palmelloid stage was seen.

Gymnodinium aeruginosum F.Stein Figs 32–35

Gymnodinium aeruginosum F.Stein 1883: pl. II, figs 19–22. – New record for the region.

LOCALITIES: **BL**: *30*, *31*, *32*.

This is a phagotrophic species that derives its functional plastids from engulphed cryptophytes and therefore displays a variable blue or greenish-blue colour (e.g., Schnepf & Elbrächter 1999). The shape of the cells is somewhat variable (Figs 32–35). Found regularly from mid-autumn to early spring.

Gymnodinium albulum Er.Lindemann

Gymnodinium albulum Er.Lindemann 1928b: 292, figs 8–10. – Nauwerck (1962).

LOCALITIES: **Mi**: *1*; **BA**: *11*, *12*.

Gymnodinium eurytopum Skuja

Gymnodinium eurytopum Skuja 1948: 368, pl. XXXVIII, figs 35, 36. – Nauwerck (1962), Oliveira (1984b).

LOCALITIES: **BA**: *14*; **AAl**: *56*.

Gymnodinium fuscum (Ehrenberg) F.Stein

BASIONYM: *Peridinium fuscum* Ehrenberg 1834: 270.

Gymnodinium fuscum (Ehrenberg) F.Stein 1878: 95. – Nauwerck (1962).

LOCALITIES: **BA**: *10**, *14*.

We recorded this fairly large and characteristic species from a small, clear water reservoir at 1450-m altitude in April 2011.

Gymnodinium lacustre J.Schiller

Gymnodinium lacustre J.Schiller 1932: 374, fig. 383. – Nauwerck (1959, 1962), Oliveira (1992).

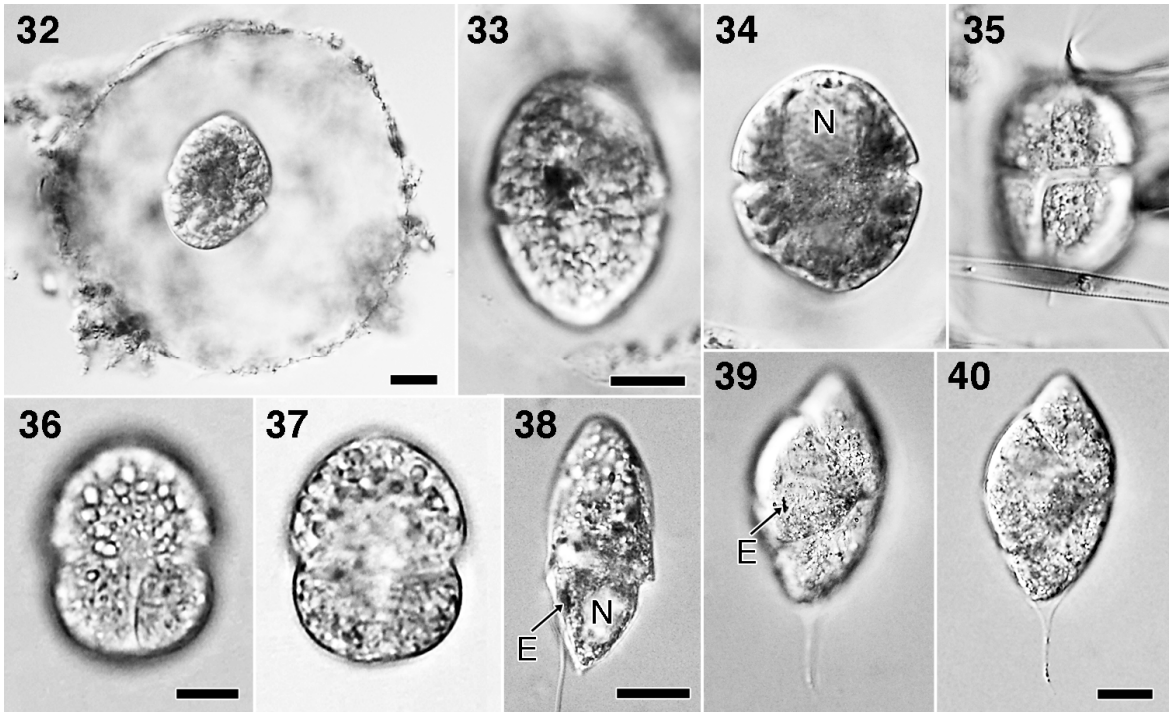
LOCALITIES: **BA**: *12*; **BL**: *42*; **BAL**: *63*.

Gymnodinium latum Skuja Figs 36–37

Gymnodinium latum Skuja 1948: 369, pl. XXXVIII, figs 42–45. – New record for the region.

LOCALITIES: **BL**: *31*.

The slowly revolving, swimming cells of this colourless species showed a very slight dorsoventral compression and a small but distinct apical protuberance. No signs of ingested material were seen in any cell. Recorded a single time in November.



Figs 32–40. LM of *Gymnodinium aeruginosum*, *G. latum* and *Opisthoaulax fastigata*. Figs 32–33. Living cells of *G. aeruginosum* from Pateira de Fermentelos (*32*), February 2011. In Fig. 32. the cell is involved by a well defined, thick mucilage layer. Figs 34–35. Living cells of *G. aeruginosum,* with somewhat different shape, collected from a pond in Gafanha da Boavista (*31*), March 2011. Same scale as Fig. 33. Figs 36–37. High focus and optical section, respectively, of live, colourless cells of *G. latum* found in November 2010 in a Gafanha da Boavista pond (*31*). Both at the same scale. Figs 38–40. Live cells of *Opisthoaulax fastigata* from Gafanha da Boavista (*31*), September 2011. Fig. 38. Cell with the longitudinal flagellum showing the eyespot (E) and the nucleus (N) in the hypocone. Figs 39–40. Non-motile, dividing stage with four forming zoospores, with elongated antapical end of sporangial cell in focus in Fig. 40. E, eyespot. Both at the same scale. All scale bars = 10 µm.

Gymnodinium mirabile Penard

Gymnodinium mirabile Penard 1891: 56, pl. V, figs 1–7. – Nauwerck (1962).

LOCALITIES: **TM**: *6**; **BA**: *11*, *12*, *15**.

This species was originally described from rather large cells (up to 90 µm long; Penard 1891). We follow Hansen & Flaim (2007) in including in this species smaller cells, down to around 50 µm long and with a more globose shape than the larger cells, which correspond approximately to *G. mirabile* var. *rufescens* Penard (1891: 57, pl. V, figs 8, 9). Portuguese records are from spring and early summer, mostly from mountain ponds above 1000 m.

Gymnodinium oligoplacatum Skuja

Gymnodinium oligoplacatum Skuja 1956: 358, pl. LXI, figs 33–34. – Nauwerck (1959).

LOCALITIES: **BL**: *42*.

Gymnodinium profundum J.Schiller

Gymnodinium profundum J.Schiller 1932: 399, fig. 416. – Nauwerck (1962).

LOCALITIES: **R**: *49*.

This species is morphologically very similar to *Gymnodinium albulum* Er.Lindemann, which seems to differ from *G. profundum* only in having been originally described without chloroplasts (Lindemann 1928b).

Gymnodinium simile Skuja

Gymnodinium simile Skuja 1956: 359, pl. LXI, figs 36–38. – Nauwerck (1962: pl. 7, fig. 18).

LOCALITIES: **BA**: *39*.

The description and illustrations given seem to represent this small species, although Nauwerck (1962) marks his identification as uncertain due to the difficulty of ascertaining some characters in the fixed material available to him.

Gymnodinium tatricum Wołoszyńska

Gymnodinium tatricum Wołoszyńska 1919: 198. – Nauwerck (1962).

LOCALITIES: **BA**: *11*.

Gymnodinium uberrimum (G.J.Allman) Kofoid et Swezy, *nomen dubium*

BASIONYM: *Peridinium uberrimum* G.J.Allman 1854: 119.

Gymnodinium uberrimum (G.J.Allman) Kofoid et Swezy 1921: 264. – Nauwerck (1962), Oliveira (1984a).

LOCALITIES: **BA**: *12*; **BAl**: *63*.

Although this taxon has been placed in *Gymnodinium* since Kofoid & Swezy's (1921) monograph, several aspects of the original description suggest instead a woloszynskioid (provided we disregard as a mistake the vibratile cilia purportedly distributed over the cell surface). However, its described features are insufficient for a confident specific or even generic assignment according to current understanding of the woloszysnkioids and the name is perhaps better avoided. Although neither Nauwerck (1962) nor Oliveira (1984a) offer any details about the organisms they called *G. uberrimum*, the concept of this species as depicted in, e.g., Huber-Pestalozzi (1950), which includes an illustration of *G. mirabile* var. *rufescens* Penard, is likely to have been the one they used. Our understanding of *G. mirabile* includes forms smaller than the very large ones originally described by Penard and may encompass Penard's var. *rufescens*, as done by Hansen & Flaim (2007). Therefore, the records listed in this entry may perhaps be referred to *G. mirabile*.

Gyrodinium helveticum (Penard) Y.Takano et T.Horiguchi

BASIONYM: *Gymnodinium helveticum* Penard 1891: 58, pl. V, figs 10–16. – Nauwerck (1959, 1962).

Gyrodinium helveticum (Penard) Y.Takano et T.Horiguchi 2004: 115.

LOCALITIES: **BA**: *11*, *14*; **BL**: *42*.

This characteristic species was shown to have an eliptical apical groove and the surface ornamented with longitudinal striations (Takano & Horiguchi 2004), and to belong therefore in *Gyrodinium* as redefined by Daugbjerg et al. (2000). The affinity with the type species of *Gyrodinium* was confirmed with SSU rDNA phylogenetic analyses (Takano & Horiguchi 2004).

Gyrodinium pusillum (A.J.Schilling) Kofoid et Swezy

BASIONYM: *Gymnodinium pusillum* A.J.Schilling 1891a: 279, pl. X, fig. 15.

Gyrodinium pusillum (A.J.Schilling) Kofoid et Swezy 1921: 329.

HETEROTYPIC SYNONYM?: *Spirodinium pusillum* cf. var. *minus* Skuja 1956: 361, pl. LXI, fig. 35 ('*minor*'). – Nauwerck (1962: pl. 7, fig. 16).

LOCALITIES: **BA**: *12*.

Nauwerck (1962) had only fixed material to work with and he was therefore uncertain about the identification, which we are referring to the typical form of *G. pusillum*. Skuja's variety, based not only on a smaller size relative to the type, but also on a smaller number of chloroplasts, may be a distinct taxon, although it has never been transferred to *Gyrodinium*. A new combination in *Gyrodinium* would now be unjustified, as the species does not show the features presently ascribed to that genus (see comment under *G. helveticum*). Re-examination of *G. pusillum* by modern methods is necessary to ascertain its phylogenetic affinities.

Jadwigia applanata Moestrup, K.Lindberg & Daugbjerg

Jadwigia applanata Moestrup, K.Lindberg & Daugbjerg in Lindberg et al. 2005: 432, figs 39–69.

MISAPPLIED NAME: *Gymnodinium neglectum* (A.J.Schilling) Er.Lindemann 1928a: 259 (Basionym: *Glenodinium neglectum* A.J.Schilling 1891a: 284, pl. X, fig. 17). – Nauwerck (1959).

LOCALITIES: **BL**: *42*.

Glenodinium neglectum is currently regarded as a woloszynskioid, usually under the name *Woloszynskia neglecta* (A.J.Schilling) R.H.Thompson (1951: 290). The common interpretation of the taxon around 1959 (e.g., Schiller 1932, Huber-Pestalozzi 1950) stemmed mainly from Wołoszyńska (1917) and Lindemann (1929), and we assume that this would have been the interpretation used by Nauwerck (1959). However, Lindberg et al. (2005) discussed the original and later illustrations of *Gymnodinium neglectum*, or *Woloszynskia neglecta*, and concluded that two species have been represented under these names. They proposed that Wołoszyńska (1917) and Lindemann (1929) described a species different from Schilling's *G. neglectum* and proposed for it a new species in the

new genus *Jadwigia* Moestrup, K.Lindberg et Daugbjerg (Lindberg et al. 2005). The ultrastructure of *J. applanata*, the type of *Jadwigia*, with an extraplastidial eyespot made up of oil globules or layers, establishes it as a member of the Tovelliaceae Moestrup, K.Lindberg et Daugbjerg.

Opisthoaulax fastigata (B.Kirchhoff et Barbara Meyer) Calado Figs 38–40

BASIONYM: *Katodinium fastigatum* B.Kirchhoff et Barbara Meyer 1995: 181, figs 1–12.

Opisthoaulax fastigata (B.Kirchhoff et Barbara Meyer) Calado 2011: 647. – New record for the region.

LOCALITIES: **BL**: *31*.

Recorded only in September 2011. (See entry on *O. vorticella* concerning the genus *Opisthoaulax* Calado.)

Opisthoaulax vorticella (F.Stein) Calado

BASIONYM: *Gymnodinium vorticella* F.Stein 1878: 90, 95; 1883: pl. III, figs 1–4.

Opisthoaulax vorticella (F.Stein) Calado 2011: 647. – Calado (2011: figs 6–10).

LOCALITIES: **BL**: *22**, *25*.

This species is known as *Katodinium vorticella* (F.Stein) A.R.Loeblich (1965: 16) in recent floras (Starmach 1974, Popovský & Pfiester 1990). The cells are phagotrophic and have a prominent, trough-shaped eyespot in the anterior part of the sulcal area. Fine-structural observations showed that the eyespot is made of electron-opaque lipid globules not bounded together by a membrane and revealed details of the organization of the flagellar base area typical of the Tovelliaceae (Calado 2011). As the type species of *Katodinium* Fott is morphologically very different and does not show tovelliacean characters the new genus *Opisthoaulax* Calado was established to accomodate a group of freshwater, phagotrophic species with a prominent eyespot resembling that of *O. vorticella* (Calado 2011).

Palatinus apiculatus (Ehrenberg) Craveiro, Calado, Daugbjerg et Moestrup

BASIONYM: *Glenodinium apiculatum* Ehrenberg 1838: 258, pl. XXII, fig. XXIV.

Palatinus apiculatus (Ehrenberg) Craveiro, Calado, Daugbjerg et Moestrup 2009: 1178. – Craveiro et al. (2009: figs 2, 4, 5).

LOCALITIES: **BL**: *31*, *32**, *34**, *35**, *36**, *37**.

This species has been mainly known as *Peridinium palatinum* Lauterborn (1896: 17) since Lefèvre's monograph (Lefèvre 1932), although the synonymy with Ehrenberg's *Glenodinium apiculatum* has been generally acknowledged. The taxonomic history of this group of peridinioids was explained in Craveiro et al. (2009). The genus *Palatinus* Craveiro, Calado, Daugbjerg et Moestrup was segregated from *Peridinium* sensu stricto on the basis of differences in the tabulation of epitheca and cingulum, in plate ornamentation, in the mode of ecdysis and in cell fine structure (Craveiro et al. 2009).

Parvodinium inconspicuum (Lemmermann) Carty

BASIONYM: *Peridinium inconspicuum* Lemmermann 1899: 350. – Nauwerck (1959, 1962), Oliveira (1982a, 1982b, 1982c, 1984a, 1984c, 1985, 1992).

Parvodinium inconspicuum (Lemmermann) Carty 2008: 106.

LOCALITIES: **Mi**: *1*, *2*, *3*; **TM**: *5*; **BB**: *18*; **BL**: *26**, *31**, *34**, *35**, *37**, *39**, *41*; **R**: *49*; **AAl**: 53; **BAl**: *58*, *59*, *62*, *63*; **Ag**: *64*, *65*.

This species is closely related to *P. umbonatum* and the boundary between the two species is difficult to define. Indeed, *P. inconspicuum* was treated as a synonym of *P. umbonatum* by Popovský & Pfiester (1986, 1990). However, populations referable to either of these species are widespread and vary somewhat in cell size, in epithecal shape and in number, size and location of hypothecal spines, suggesting that more than one species is involved. Previous records of *P. inconspicuum* were not accompanied by any descriptive data supporting identification and we must assume they refer to populations of small cells (down to about 15 µm long) or with flat or concave plates on the epitheca (e.g., Huber-Pestalozzi 1950). We used the same general guidelines for the new records presented above. The illustrations given by Craveiro & Santos (1999) for *P. umbonatum* (and reproduced again in Santos et al. 2002) are perhaps referable to *P. inconspicuum*. Correlative studies of genetic and morphological variability in this group of species are needed to clarify species boundaries. (See entry on *P. umbonatum* concerning the genus *Parvodinium* Carty.)

Parvodinium pusillum (Penard) Carty

BASIONYM: *Glenodinium pusillum* Penard 1891: 52, pl. IV, figs 1–4.

Peridinium pusillum (Penard) Lemmermann 1901: 65. – Nauwerck (1962: pl. 7, fig, 17), Oliveira (1982b).

Parvodinium pusillum (Penard) Carty 2008: 106.

LOCALITIES: **BA**: *11*, *12*, *14*; **BB**: *17*; **BL**: *38*; **R**: *49*; **AAl**: *51*; **BAl**: *58*.

Popovský & Pfiester (1986, 1990) regarded *Peridinium pusillum* as a synonym of *P. umbonatum*. However, in *P. pusillum* the sulcal groove is much smaller and does not widen toward the antapex as in *P. umbonatum*, indicating a distinct species.

Parvodinium umbonatum (F.Stein) Carty

BASIONYM: *Peridinium umbonatum* F.Stein 1883: pl. XII, figs 1–8. – Calado (1993), Calado & Craveiro (1993), Craveiro & Santos (1998, 1999: pl. I, fig. 16), Santos et al. (2002: pl. 4, figs 12, 13).

Parvodinium umbonatum (F.Stein) Carty 2008: 106.

LOCALITIES: **BA**: *11**; **BL**: *31**, *40*, *41*, *45*.

Species with an apical pore, two anterior intercalary plates on the dorsal side of the epitheca and six plates in the cingulum, which made up *Peridinium* group umbonatum in earlier classifications (Huber-Pestalozzi 1950, Starmach 1974, Popovský & Pfiester 1990), were moved to the separate genus *Parvodinium* Carty (2008: 106). (See also the entry on *P. inconspicuum*.)

Peridiniopsis cunningtonii Lemmermann

Peridiniopsis cunningtonii Lemmermann in West 1907: 189, pl. 9, fig. 2. – Calado & Craveiro (1993).

LOCALITIES: **BL**: *31**, *34**, *37*.

Recorded sporadically in early summer and in autumn.

Peridiniopsis elpatiewskyi (Ostenfeld) Bourrelly Figs 41–44

BASIONYM: *Peridinium umbonatum* var. *elpatiewskyi* Ostenfeld 1907: 391, pl. IX, figs 9–12.

Peridiniopsis elpatiewskyi (Ostenfeld) Bourrelly 1968: 9. – New record for the region.

LOCALITIES: **BL**: *26*, *31*, *34*, *37*.

This name is here used in the sense of Lefèvre (1932) and later workers, which apparently differs from Ostenfeld's (1907) original description. To preserve current usage, Meyer & Elbrächter (1996) proposed conservation of the name *Peridinium elpatiewskyi* with the expressed intention that conservation would extend to the basionym *P. umbonatum* var. *elpatiewskyi* Ostenfeld. However, the actual entry in the Botanical Code (McNeill et al. 2006) for the conserved *Peridinium elpatiewskyi* Lemmermann (sic) excludes any reference to the basionym, rendering it useless outside *Peridinium*. The species was found in spring and early summer, and in early autumn, sometimes as a prominent member of the plankton.

Peridiniopsis polonica (Wołoszyńska) Bourrelly

BASIONYM: *Peridinium polonicum* Wołoszyńska 1916: 271, pl. 12, figs 1–10.

Peridiniopsis polonica (Wołoszyńska) Bourrelly 1968: 9. – Oliveira (1984b).

LOCALITIES: **BL**: *31**, *56*.

A peridinioid species that commonly appears as a sister group to a clade with marine species of *Scrippsiella*, both based on SSU rDNA (Logares et al. 2007) and on LSU rDNA (Craveiro et al. 2011); it is therefore unlikely to be a member of either *Peridinium* or *Peridiniopsis*. Fine structural analysis is being conducted to define the taxonomic position of *P. polonica* (Craveiro et al., in prep.). The earlier name *Glenodinium gymnodinium* Penard (1891: 54, pl. IV, figs 8–10) is often considered synonymous with *P. polonica*, in which case the epithet *'gymnodinium'* has priority (contrast with, e.g., Popovský & Pfiester 1990). We recorded the species half a dozen times in the autumn and late winter.

Peridinium aciculiferum Lemmermann

Peridinium aciculiferum Lemmermann 1900: 28. – Nauwerck (1959).

LOCALITIES: **BL**: *42*.

Reported from samples collected in February and early April by M. Póvoa dos Reis (Nauwerck 1959). *Peridinium aciculiferum* is a cold water species (Rengefors & Anderson 1998, Hansen & Flaim 2007) and is perhaps under-represented for lack of sampling in suitable places during the winter months.

Peridinium cinctum (O.F.Müller) Ehrenberg

BASIONYM: *Vorticella cincta* O.F.Müller 1773: 98.

Peridinium cinctum (O.F.Müller) Ehrenberg 1830: 38. – Nauwerck (1962), Oliveira (1984a, 1984b, 1984c), Calado et al. (1991), Calado & Craveiro (1993), Calado & Craveiro (1996), Santos et al. (1996), Calado et al. (1999), Santos et al. (2002).

LOCALITIES: **BA**: *8**; **BL**: *27*, *30*, *31**, *32*, *33**, *34**, *35**, *37*, *39*, *40*, *42*, *46*; **R**: *49*; **AAl**: *53*, *56*; **BAl**: *59*, *61*, *63*; **Ag**: *64*, *65*.

A fairly large and conspicuous species, common in meso- to eutrophic ponds and shallow lakes throughout the region.

Peridinium cinctum f. ***maeandricum*** M.Lefèvre Figs 45–47

Peridinium cinctum f. *maeandricum* M.Lefèvre 1926: 334, pl. XII, figs 1–4 ('*meandricum*'). – New record for the region.

LOCALITIES: **AAl**: *55*.

Recorded only once, on 23 March 2008. It was abundant in the sample and all cells showed the characteristic vermicular ridges on at least some of the hypothecal plates.

Peridinium raciborskii Wołoszyńska

Peridinium raciborskii Wołoszyńska 1912: 700, text-fig. 21.

Peridinium palustre var. *raciborskii* (Wołoszyńska) M.Lefèvre 1932: 99. – Lacerda (1948: pl. II, fig. 29).

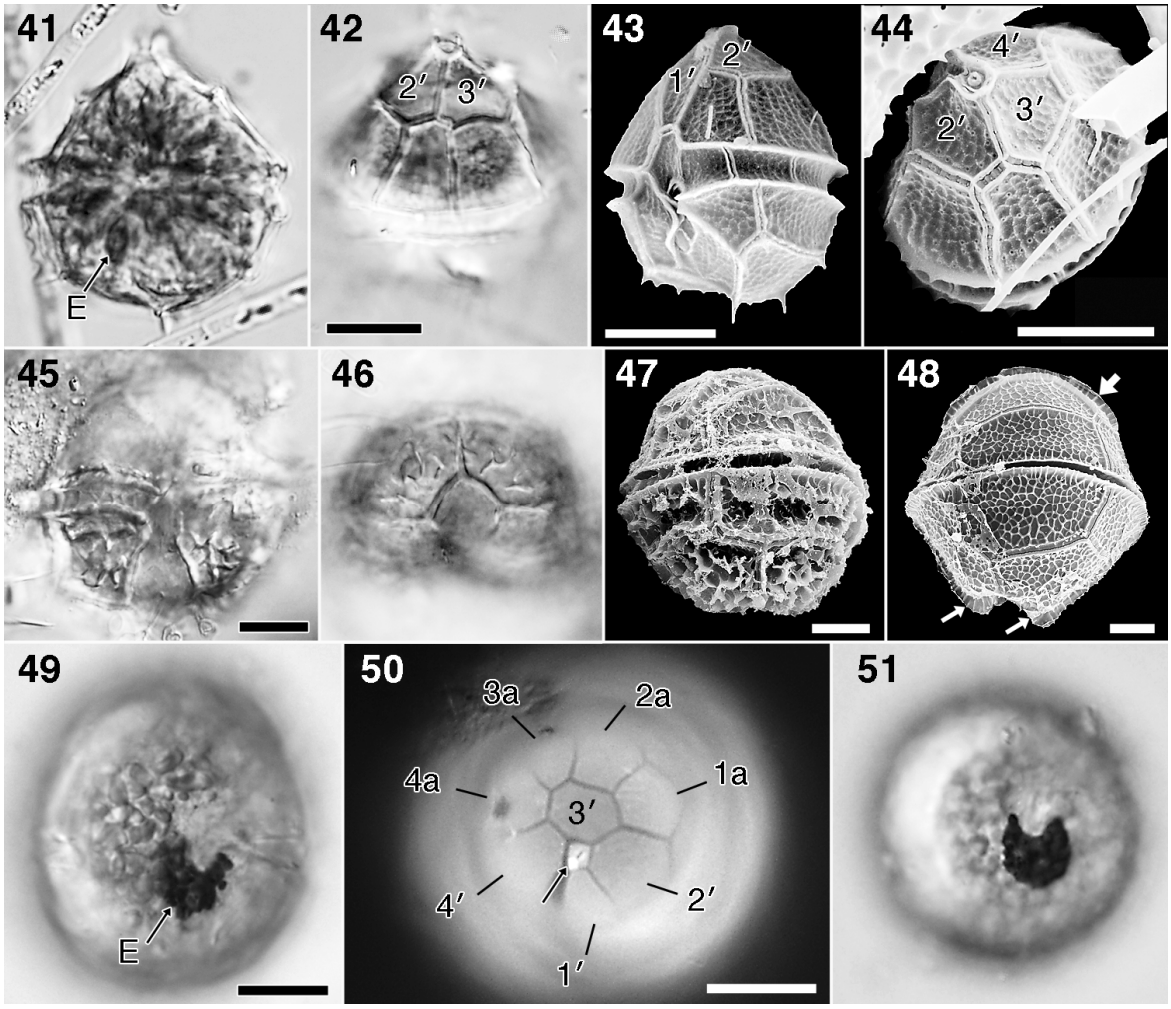
LOCALITIES: **BL**: *44*.

Lacerda (1948) gives a side view of a dorsoventrally flattened cell with the apical plate asymmetry typical of group cinctum. His description of a bilobed hypotheca terminating in two short horns fits *P. raciborskii*, although the cells, not exceeding 50 µm, were rather small.

Peridinium volzii Lemmermann

Peridinium volzii Lemmermann 1905: 166, pl. XI, figs 15–18. – Nauwerck (1959, 1962), Oliveira (1982a, 1982b), Vasconcelos (1991).

LOCALITIES: **Mi**: *1*; **TM**: *7*; **BA**: *14*; **BL**: *42*, *47*.



Figs 41–51. *Peridiniopsis elpatiewskyi*, *Peridinium cinctum* f. *maeandricum*, *P. willei* and *Sphaerodinium cracoviense* from field samples. Figs 41–42. LM of live cells of *Peridiniopsis elpatiewskyi* from a pond in Gafanha da Boavista (*31*), May 2011. Note the eyespot (E) in the sulcus and the absence of intercalary plates in dorsal view. Both at the same scale. Figs 43–44. SEM of *P. elpatiewskyi* from the same location (*31*), October 2006. In the apical-dorsal view of Fig. 44 apical plates that dorsally surround the pore are visible in direct contact with precingular plates. Figs 45–46. LM of fixed cells of *Peridinium cinctum* f. *maeandricum* from a puddle near Montemor-o-Novo (*55*), March 2008. Ventral and antapical views, respectively. Note the vermicular hypotheca, typical of this forma. Both at the same scale. Fig. 47. Dorsal view of *P. cinctum* f. *maeandricum* from the same sample. SEM. Fig. 48. Dorsal view of *Peridinium willei*, SEM. From Pateira de Fermentelos (*32*), January 2006. Note the characteristic flanges on the hypotheca (thinner arrows) and on the epitheca (thick arrow). Figs 49–50. *Sphaerodinium cracoviense* from Parque da Balsa (*29*), June 2010. Fig. 49. Ventral view of a cell with a large eyespot (E). Fig. 50. Fluorescence microscopy of CalcoFluor White-stained epitheca showing the four apical plates around the apical pore (arrow) and four intercalary plates. Fig. 51. LM of *S. cracoviense* from Ribeiro da Palha (*34*), November 2010, with a very large typical eyespot with a more defined margin. Same scale as Fig. 49. All scale bars = 10 µm.

Peridinium willei Huitfeldt-Kaas Fig. 48

Peridinium willei Huitfeldt-Kaas 1900: 5, figs 6–9. – New record for the region.

LOCALITIES: **BA**: *11*; **BL**: *32*, *38*

This large and very characteristic *Peridinium* is apparently uncommon in Portugal. We recorded it only four times in the coastal region in the winter months, and in Serra da Estrela at 1500 m in April.

Prosoaulax lacustris (F.Stein) Calado et Moestrup

BASIONYM: *Amphidinium lacustre* F.Stein 1883: 15, pl. XVII, figs 21–30. – Nauwerck (1962: pl. 7, fig. 20)?

Prosoaulax lacustris (F.Stein) Calado et Moestrup 2005: 113.

HETEROTYPIC SYNONYMS: *Amphidinium tatrae* Wołoszyńska 1937: 190, pl. IX, fig. 5. – Nauwerck (1962).

Amphidinium luteum Skuja 1939: 148, pl. X, figs 18–20. – Nauwerck (1959).

LOCALITY: **BA**: *12*; **BL**: *32**, *36**.

The changes in the taxonomic concept of *Amphidinium lacustre* induced by Schilling's (1891a, 1913) interpretation of the taxon were discussed by Calado et al. (1998). Nauwerck's (1962) drawing resembles more Schilling's modification than Stein's original illustrations and is therefore considered a doubtful representation of the taxon. The proposed synonymy follows Javornický (1967) and Calado & Moestrup (2005). The genus *Prosoaulax* Calado & Moestrup (2005: 113) was established to accomodate a group of presumably related freshwater species that were removed from *Amphidinium* following its redefinition by Flø Jørgensen et al. (2004). The fine structure of the type species, *P. lacustris*, especially the presence of an eyespot containing brick-like material, suggests affinity with the Suessiales (Calado & Moestrup 2005).

Prosoaulax multiplex (J.Schiller) Calado et Moestrup

BASIONYM: *Amphidinium multiplex* J.Schiller 1955: 23, pl. I, fig. 7.

Prosoaulax multiplex (J.Schiller) Calado et Moestrup 2005: 115.

HETEROTYPIC SYNONYM: *Amphidinium lohammarii* Skuja 1956: 354, pl. LXI, figs 18–22. – Nauwerck (1962).

LOCALITY: **R**: *49*.

The synonymy follows Calado & Moestrup (2005), where the species was interpreted as distinct from *Prosoaulax lacustris* because the sulcus in *P. multiplex* extends onto the epicone.

Sphaerodinium cracoviense Wołoszyńska Figs 49–51

Sphaerodinium cracoviense Wołoszyńska 1916: 281, pl. 14, figs 28–30. – New record for the region.

LOCALITIES: **BL**: *29*, *31*, *32*, *34*, *35*.

Despite its superficial resemblance to the peridinioids, this peculiar species is more closely related to the woloszynskioids (Craveiro et al. 2010). Its large eyespot was shown to have a unique structure that combines a vesicle containing brick-like units with 1–3 underlying oil layers not bounded by a membrane; these features suggest affinity with, respectively, the Suessiales or the Tovelliaceae. Details of flagellar apparatus and pusule structure, and the presence of a membranous lamellar body point to a position in or near the Suessiales (Craveiro et al. 2010). We found *Sphaerodinium* irregularly in the Aveiro region nearly all year round. Although live cells from different samples appeared similar in the light microscope they were not always examined in detail to ascertain species identity, so the possibility that other *Sphaerodinium* species were present cannot be ruled out.

Tyrannodinium edax (A.J.Schilling) Calado

BASIONYM: *Glenodinium edax* A.J.Schilling 1891b: 206, 207, pl. X, figs 23, 24. – Nauwerck (1959).

Tyrannodinium edax (A.J.Schilling) Calado 2011: 643. – Calado (2011: figs 2, 3).

HETEROTYPIC SYNONYMS: *Peridiniopsis berolinensis* (Lemmermann) Bourrelly 1968: 9. – Calado & Moestrup (1997).

Tyrannodinium berolinense (Lemmermann) Calado, Craveiro, Daugbjerg et Moestrup 2009: 1203. – Calado et al. (2009: figs 1–5).

LOCALITIES: **BL**: *23*, *24*, *26**, *30*, 31**, *32**, *33**, *34**, *35**, *36**, *37**, *38**, *39**, *42*.

This predatory species is very common in the Aveiro region year round and it is likely that examination of samples from mesotrophic and eutrophic ponds in other areas will

reveal a wider distribution. It is a freshwater member of the Pfiesteriaceae Steidinger et J.M.Burkholder (Calado et al. 2009). The synonymy follows Calado (2011).

Woloszynskia ordinata (Skuja) R.H.Thompson

BASIONYM: *Gymnodinium ordinatum* Skuja 1939: 151, pl. X, figs 26–28. – Nauwerck (1959).

Woloszynskia ordinata (Skuja) R.H.Thompson 1951: 291.

LOCALITIES: **BL**: *42*.

Although this species is currently classified in the genus *Woloszynskia* (e.g., Starmach 1974, Popovský & Pfiester 1990) its phylogenetic affinities within the heterogenous group of recently described woloszynskioid genera need to be reexamined.

Woloszynskia pascheri (Suchlandt) Stosch

BASIONYM: *Glenodinium pascheri* Suchlandt 1916: 246, text-figs A–F.

Gyrodinium pascheri (Suchlandt) Er.Lindemann 1928a: 259. – Nauwerck (1959, 1962: pl. 7, fig. 15).

Woloszynskia pascheri (Suchlandt) Stosch 1973: 133.

LOCALITIES: **BA**: *11*, *12*; **BL**: *42*.

As with other dinoflagellates with a delicate cell cover, Nauwerck's access to fixed material alone made the identification uncertain. The illustrations given in Nauwerck (1962) are perhaps compatible with the original description, although they show a slightly more helical cingulum than Suchlandt's (1916) drawings. The species was described to accumulate carmine-red pigment and to form red patches in snow and ice. The large morphological variability ascribed to *W. pascheri* in Popovský & Pfiester (1990) is perhaps the result of confusion with other species capable of storing red pigments. Until it is examined by modern methods the identity, variability and phylogenetic affinities of this species are uncertain.

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CHAPTER 3

STUDIES ON WOLOSZYNSKIOID DINOFLAGELLATES VI: DESCRIPTION OF *TOVELLIA AVEIRENSIS* SP. NOV. (DINOPHYCEAE), A NEW SPECIES OF TOVELLIACEAE WITH SPINY CYSTS

Pandeirada, M.S., Craveiro, S.C., Daugbjerg, N., Moestrup, Ø. & Calado, A.J. Studies on woloszynskioid dinoflagellates VI: description of *Tovellia aveirensis* sp. nov. (Dinophyceae), a new species of Tovelliaceae with spiny cysts. *European Journal of Phycology* (submitted on 7 November 2013) – Inclusion in this thesis is not intended as effective publication of the new taxon proposed in the manuscript

ABSTRACT

A new species of *Tovellia*, *T. aveirensis*, is described on the basis of light (LM) and scanning electron microscopy (SEM) of motile cells and resting cysts, complemented with transmission electron microscopy (TEM) of flagellate cells, and a phylogenetic analysis built from partial sequences of the large subunit ribosomal rRNA gene. Both vegetative cells and several stages of a life cycle involving sexual reproduction and the production of resting cysts were examined in cultures established from a tank in the University of Aveiro campus. Vegetative cells were round and little compressed dorsoventrally, planozygotes were longer and had a proportionally larger epicone. Chloroplast lobes were shown by TEM to radiate from a central, branched pyrenoid, although this was difficult to ascertain in LM. The amphiesma of flagellate cells had mainly five or six-sided vesicles with thin plates, arranged in 5–7 latitudinal series on the epicone, 3–5 on the hypocone. The cingulum had two rows of plates, the posterior row extending into the hypocone and crossed by a series of small projecting knobs along the lower edge of the cingulum. A line of narrow amphiesmal plates (ALP) extended over the cell apex, from near the cingulum on the ventral side to near the middle of the dorsal side of the epicone. Eight or nine narrow amphiesmal plates lined each side of the ALP. Resting cysts differed from any described before in having numerous long, tapering spines with branched tips distributed over most of the surface. Most mature cysts showed an equatorial constriction. Neither cysts nor motile cells were seen to accumulate red cytoplasmic bodies in any stage of the cultures. The phylogenetic analysis placed, with high statistical support, the new species within the genus *Tovellia*; it formed a clade with *T. sanguinea*, a species notable for its reddening cells, with moderate support.

Key words: cyst, dinoflagellates, LSU rDNA, phylogeny, taxonomy, Tovelliaceae, *Tovellia aveirensis*, ultrastructure

INTRODUCTION

Recent studies on the so-called woloszynskioid dinoflagellates, traditionally characterized by having a cell cover of numerous thin amphiesmal plates, revealed a heterogeneous, polyphyletic assemblage (Lindberg *et al.,* 2005; Moestrup *et al.,* 2008).

The consequent reclassification of woloszynskioid species led to the establishment of several genera distributed over different families: *Tovellia* Moestrup, K. Lindberg & Daugbjerg, *Jadwigia* Moestrup, K. Lindberg & Daugbjerg, *Esoptrodinium* Javornický (a probable synonym of *Bernardinium* Chodat) and *Opisthoaulax* Calado are currently included in the family Tovelliaceae (Lindberg *et al.,* 2005; Calado *et al.*, 2006; Calado, 2011; Fawcett & Parrow, 2012); *Borghiella* Moestrup, Gert Hansen & Daugbjerg and *Baldinia* Gert Hansen & Daugbjerg are placed in the Borghiellaceae (Hansen *et al.,* 2007; Moestrup *et al.,* 2008, 2009*a*); *Biecheleria* Moestrup, K. Lindberg & Daugbjerg and *Biecheleriopsis* Moestrup, K. Lindberg & Daugbjerg are ranged with the Suessiaceae (Moestrup *et al.,* 2009*a*, 2009*b*). These extensive taxonomic changes are supported by molecular data and by morphological differences in eyespot structure, organization of the cell apex and type of resting cyst.

Members of the Tovelliaceae typically possess an extraplastidial eyespot composed of pigment globules not bounded by membranes (eyespot type C in Moestrup & Daugbjerg, 2007). In addition, in *Tovellia* and *Jadwigia* a straight or slightly curved line of narrow plates provided with a row of knobs (ALP *sensu* Lindberg *et al.*, 2005) is present on the epicone and lined on each side by a row of amphiesmal plates narrower than the average hexagonal or pentagonal vesicles of the amphiesma. *Tovellia* species produce resting cysts with an equatorial constriction (sometimes interpreted as a paracingulum), axial horns and pre- and postcingular protuberances or scattered short spines; cysts with equatorial constriction and axial horns have also been demonstrated in *Opisthoaulax vorticella* (F. Stein) Calado (Lindberg *et al.*, 2005; Moestrup *et al.*, 2006; Calado 2011). In contrast *Jadwigia* and *Esoptrodinium/Bernardinium* produce smooth, round resting cysts (Lindberg *et al.,* 2005; Calado *et al.*, 2006).

The present work describes a new species of *Tovellia* with a particular type of cyst, slightly constricted in the middle and ornamented by numerous processes that are variously branched near the tip. The morphology of swimming cells and cysts is described on the basis of light (LM) and scanning electron microscopy (SEM), and the general internal fine structure of swarmers is shown in transmission electron microscopy (TEM). A phylogenetic analysis based on partial nuclear-encoded large subunit ribosomal RNA gene sequences (LSU rDNA) corroborates the taxonomic assignment of the new species. This is the first species of *Tovellia* to be reported from Portugal (Pandeirada *et al.*, 2013).

MATERIAL AND METHODS

Biological material

The organism described in this work was found in a net sample (mesh size 25-30 µm) collected from a clean water tank at the University of Aveiro Campus, Aveiro, Portugal, on 12 October 2009. Several swimming cells were transferred to one culture well with L16 medium (Lindström, 1991) supplemented with vitamins according to Popovský & Pfiester (1990) and maintained at 18ºC with 12:12 light:dark photoperiod. One month later, five cysts were observed in the well. These were re-isolated separately to five wells with the same medium and placed under the same temperature and light conditions. Three cysts germinated and gave origin to three culture lines.

Light microscopy (LM)

Light micrographs of motile cells and cysts were taken with a Zeiss Axioplan 2 imaging light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a DP70 and a ColorView IIIu Olympus cameras (Olympus Corp., Tokyo, Japan). Asexual reproduction was recorded with a JVC color video camera mounted on a Leitz Biomed light microscope (Leica Microsystems, Wetzlar, Germany). Images of this process were prepared from still frames of the recorded videos.

Scanning electron microscopy (SEM)

A clear visualization of amphiesmal vesicles (by removing the outer membrane) of the swimming cells was obtained with the following procedure: 1.5 ml of culture was fixed for 25 min by adding 1 ml of a fixative mixture made of saturated $HgCl_2$ and 2% osmium tetroxide in a proportion of 1:5. After fixation, cells were retained on Isopore polycarbonate filters with 8-µm pore size (Millipore Corp., Billerica, USA) and washed with distilled water for 10 min. Dehydratation of filters with the cells was performed with a graded ethanol series with an overnight stop in 70% ethanol at 4ºC. Dehydration was completed the following day and the material was critical-point-dried in a Baltec CPD-030 (Balzers, Liechtenstein). The dried filters were glued onto stubs, sputter-coated with gold-palladium and examined with a Hitachi S-4100 (Hitachi High-Technologies Corp., Tokyo, Japan) scanning electron microscope.

Cysts were prepared in a similar way with a different fixative proportion: 1 ml of culture was added to 0.5 ml of fixative mixture made of saturated $HgCl_2$ and 2% osmium tetroxide in a proportion of 1:3, for 15 min.

Transmission electron microscopy (TEM)

Swimming cells from culture were picked up, transferred to 2% glutaraldehyde in phosphate buffer 0.1 M, pH 7.2, and fixed for 1 h 15 min. After being washed in the same buffer, cells were embedded in 1.5% agar blocks and post-fixed overnight in 0.5% osmium tetroxide in the same buffer. The agar blocks with the cells were rinsed in phosphate buffer and distilled water. Following dehydration through a graded ethanol series and propylene oxide they were embedded in Spurr's resin. The resin blocks were cured for about 14 h at 70ºC. Cells were sectioned with a diamond knife on a EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Ribbons of sections 70 nm thick were picked up with slot grids and placed on Formvar film. Sections were stained with uranyl acetate and lead citrate and examined using a Zeiss EM 10A (Carl Zeiss, Oberkochen, Germany) transmission electron microscope operated at 60 kV.

DNA extraction and PCR amplification of LSU rDNA

Ten ml of exponentially growing culture line MSP14c was harvested by centrifugation at 1201 g for 10 min. Most of the supernatant was discarded and the cell pellet resuspended in about 100 µl growth media. The material was transferred to a 1.5 ml Eppendorf tube and allowed to freeze for 2 days at –18 °C. After this time 30 µl of the cell pellet was extracted using the CTAB method of Doyle & Doyle (1987) with a few modifications as previously outlined by Daugbjerg et al. (1994). PCR amplification of partial LSU rDNA (nearly 1400 base pairs) was performed as stated in Hansen & Daugbjerg (2011).

The PCR amplified fragments of LSU rDNA were purified as described in Craveiro *et al.* (2013). 30 ng of DNA was used for sequence determinations in both directions using external primers (i.e. D1R and ND28-1483R) and internal primers (i.e. D3A, D3B and D2CR). See Daugbjerg *et al.* (2013) and Scholin *et al.* (1994) for primer sequences. For sequencing we used the service provided by Macrogen, Korea.

Single cell PCR partial amplification of LSU rDNA

For comparison between culture lines, single or pairs of cells of a different strain, MSP14b, were isolated, using a micropipette under the inverted microscope, to 0.2-ml PCR tubes and frozen at -8ºC for 3 days before PCR reactions. Cell DNA constituted the template to amplify about 1500 base pairs (bp) of the LSU rRNA gene using the terminal primers D1R (Scholin *et al.,* 1994) and 28-1483R (Daugbjerg *et al.,* 2000). These were added to the PCR tubes with the isolated cells, followed by illustra^{TM} puReTaq Ready-To-Go PCR Beads (GE Healthcare UK Ltd) containing all other chemicals necessary for PCR amplification. The reaction occurred in the Biometra–Tprofessional Trio thermocycler. Thermal cycling for PCR amplification was as outlined in Moestrup *et al.* (2008), but with a longer final extension step of 10 min (rather than 6 min). DNA fragments were loaded on a 1% agarose gel, run for 20 min at 90 V and viewed under a UV light table (Molecular imager chemiDoc XRS System from Bio-Rad Laboratories, Inc.). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's recommendations, and sent to Macrogen Europe (Amsterdam, The Netherlands) for sequence determination in both directions. The sequencing primers used were D1R, D2C, D3A, D3B and 28-1483R (for primer sequences, see Scholin *et al.,* 1994; Daugbjerg *et al.,* 2000; Hansen *et al.,* 2000). The sequences obtained were identical to that of MSP14c.

Alignment and phylogeny

Sequence alignment was optimized based on the secondary structure of the LSU rRNA molecule as proposed by de Rijk *et al.* (2000) and further edited by eye using Jalview, ver. 2.8 (Waterhouse *et al.*, 2009). Our data matrix comprised 1173 base pairs including introduced gaps. This corresponded to a DNA fragment that covered variable domain D1 to 8 base pairs of the variable domain D6 (sensu Lenaers *et al.*, 1989). However, domain D2 was omitted, as this is known to be highly divergent and therefore a challenging task to align unambiguously. To infer the phylogeny of *Tovellia aveirensis* we used both Bayesian (BA) and maximum likelihood (ML) analyses. For BA we used MrBayes ver. 3.2.2 (Ronquist & Huelsenbeck, 2003) and for ML we used PhyML ver. 3.0 (Guindon & Gascuel, 2003). In BA we used 20×10^6 generations and a tree was sampled every 1000th generation. The BA analysis was run on a local computer. In order to evaluate the burn-in value we plotted the LnL values as a function of generations in a spreadsheet. The burn-in

occurred after $2{,}001\times10^3$ generations (conservative estimate), thus 2,001 trees were removed leaving 18,000 trees for generating a 50% majority-rule consensus in PAUP* ver. 4.0b10 (Swofford, 2003). For ML analysis we applied the parameter settings obtained from jModelTest (ver. 2.1.4, Darriba *et al.* (2012). PhyML (ver. 3.0) was run via the online version available on the Montpellier bioinformatics platform at http://www.atgc-montpellier.fr/phyml. The robustness of the tree topologies was evaluated using bootstrapping with 1,000 replications.

Comparative studies using ultrastructural characters and phylogenetic reconstructions based on molecular data (e.g. Van de Peer *et al.*, 1996) have indicated that Ciliata and Apicomplexa comprise the sister group to the Dinophyceae. Thus, we used four species of ciliates (*Euplotes aediculatus*, *Spathidium amphoriforme*, *Tetrahymena thermophila*, *T. pyriformis*), seven species of apicomplexans (*Cryptosporidium parvum*, *Eimeria tenella, Hammondia hammondi*, *Neospora canium*, *Sarcocystis neurona*, *Theileria parva*, *Toxoplasma gondii*) and the perkinsid *Perkinsus andrewsi* to polarize the ingroup of dinoflagellates. The ingroup consisted of a diverse assemblage of dinoflagellates representing 39 genera and 64 species.

We used PAUP* (ver. 4.0b10) to calculate the sequence divergence estimate between all pairwise comparisons involving three species of *Tovellia*, *Esoptrodinium gemma* and *Jadwigia applanata*.

RESULTS

Tovellia aveirensis Pandeirada, Craveiro, Daugbjerg, Moestrup & Calado, *sp. nov.* (Figs 1–21)

Description: Vegetative cells ovoid or nearly spherical, slightly compressed dorsoventrally or not at all. Cingulum descending, displaced about one cingulum width. Epicone broadly round, slightly longer than wide and often somewhat longer than the obliquely flattened hypocone. Cells 25–34 μm long, 17–24 μm wide and 14–21 μm thick. Chloroplast lobes yellowish-green, radiating from the cell centre towards the periphery. Nucleus in the hypocone. Eyespot nearly rectangular in ventral view, located in the sulcal area. Cell cover mainly formed by pentagonal or hexagonal amphiesmal vesicles roughly arranged in latitudinal series, 5–7 series on the epicone and 3–5 on the hypocone.

Cingulum with two series of vesicles, the anterior vesicles abutting the sharply defined anterior cingulum edge whereas the roughly hexagonal vesicles of the posterior row extend into the hypocone over the rounded posterior cingulum edge. A row of knobs marked the posterior edge of the cingulum. The initial part of the cingulum showed additional two or three nearly hexagonal vesicles intercalated between the two regular vesicle rows. A line of narrow vesicles started near the proximal end of the cingulum, on the ventral side, and extended over the apex of the cell. Cysts yellowish-brown, ornamented by branched processes that were usually absent in the equatorial area, which was often slightly constricted. Nuclear-encoded partial LSU rRNA gene sequence = GenBank accession XXXXXXX.

Holotype: SEM stub with critical point dried material from culture line MSP14c, fixed to display the amphiesmal vesicles, deposited at the University of Aveiro Herbarium registered as AVE-A-T-4. Figures 8–13 illustrate cells from this stub.

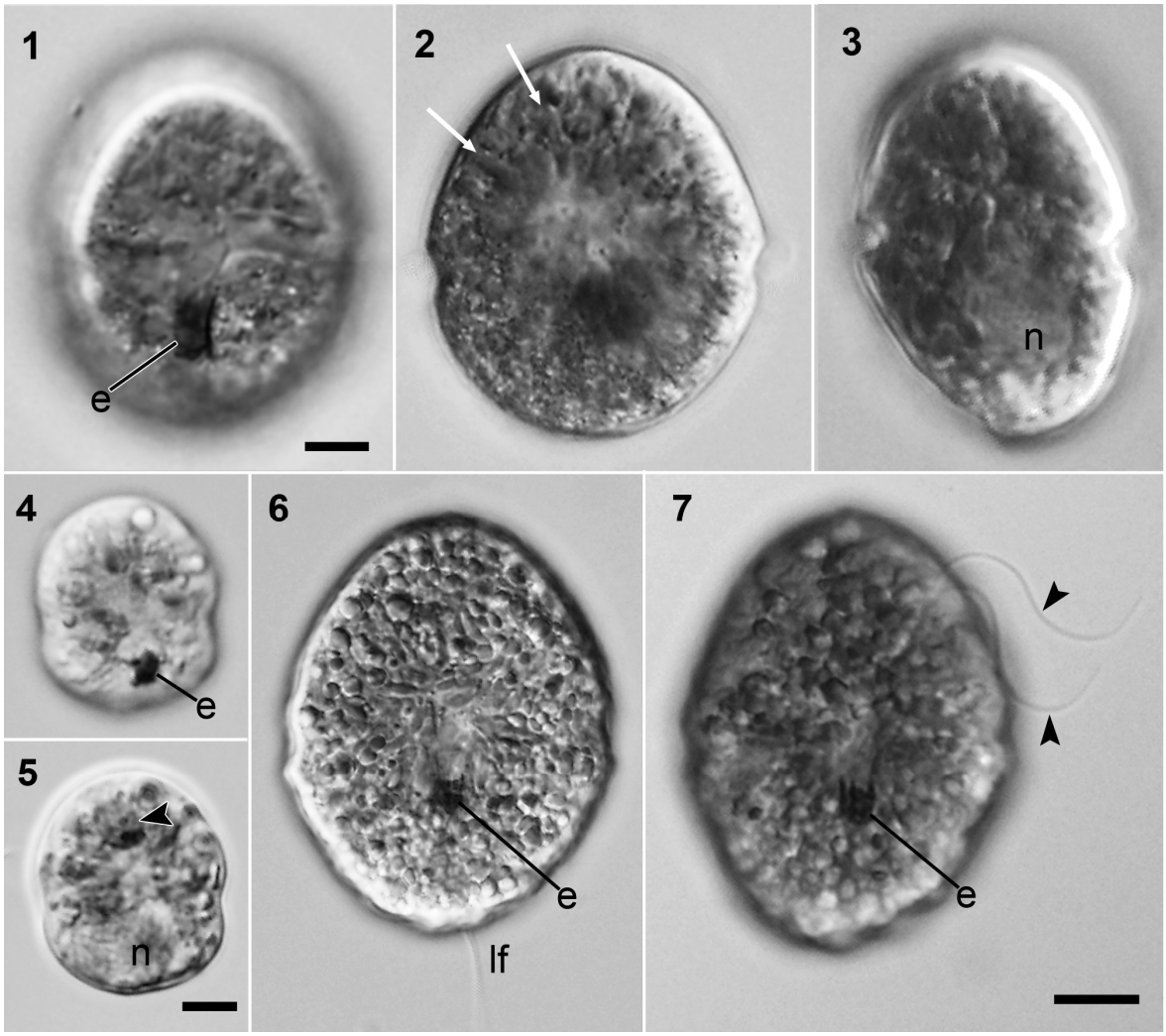
Isotype: SEM stub with critical point dried material from culture line MSP14c, containing swarmers, mostly retaining outer membranes, and mature cysts, deposited at the University of Aveiro Herbarium registered as AVE-A-T-5. Figures 22–25 illustrate cysts from this stub.

Type locality: Freshwater (conductivity about 300 µS) tank at the University of Aveiro Campus, Aveiro, Portugal (40°38'4.52"N, 8°39'30.21"W), collected on 12 October 2009.

Etymology: Latin *aveirensis*, 'from Aveiro', in reference to both the city and the University of Aveiro Campus, where the species was found.

General morphology of motile cells

Motile cells are illustrated in LM and SEM in Figs 1–12. Vegetative cells were ovoid or nearly spherical, not compressed dorsoventrally at cingulum level or only slightly so (Figs 1–3, 8–11). The cingulum was located slightly below the middle of the cell and had



Figs 1–7. *Tovellia aveirensis* sp. nov., LM. 1–3. Vegetative cells. 1. Ventral view in surface focus showing the cingulum displaced about one cingulum width and the rectangular eyespot (e) in the sulcus. 2. Optical section of the cell shown in Fig. 1 with faintly visible radial arrangement of chloroplast lobes (white arrows). 3. Lateral view showing the nucleus (n) and the slanting ventral surface of the hypocone. 4, 5. Small cell (gamete) focused in different planes showing eyespot (e), nucleus (n) and accumulation body (arrowhead). 6, 7. Planozygotes with relatively small eyespot (e). The arrowheads in Fig. 7 indicate the paired longitudinal flagella. The following Figs are at the same scale: Figs 1–3; Figs 4, 5; Figs 6, 7. All scale bars = 10 µm.

the distal (right-hand side) end about one cingulum width below the proximal end (Figs 1, 8). The epicone was broadly round and slightly longer than wide (Figs 1, 2, 8). In side view the hypocone appeared obliquely flattened (Figs 3, 10). Cells were 25–34 µm long (n = 30), 17–24 µm wide (n = 30) and 14–21 µm thick (n = 19). A bright-red, trough-shaped eyespot (rectangular in ventral view) underlay the full width of the sulcus (3–3.5 µm in the proximal half) along up to 4 µm of its length, although it was usually smaller in non-

vegetative cells (Figs 1, 4, 6, 7). The sulcus widened somewhat in its posterior half (Figs 8, 11). Vegetative cells displayed numerous yellowish-green chloroplast lobes near the surface (Figs 1, 3). A radial arragement of chloroplast lobes was barely discernible in optical sections of the epicone (Fig. 2). The roundish to transversely ellipsoid nucleus occupied a large portion of the hypocone (Figs 3, 5). Vegetative cells swam with a regular, continuous motion, usually rotating around the longitudinal axis. Smaller, roughly spherical cells, 16.5–20 μm long (n = 5), 12–15.5 μm wide (n = 5), about 12 μm thick (n = 2), commonly appeared in the cultures; they had fewer chloroplast lobes, to the point of sometimes appearing nearly colourless (Figs 4, 5), and occasional accumulation bodies (Fig. 5, arrowhead). These rapidly swimming small cells were seen fusing with one another to yield the formation of planozygotes and are therefore referred to as gametes. Planozygotes, identified by the presence of two longitudinal flagella (Fig. 7), were usually more elongated than vegetative cells (Figs 6, 7). They ranged between 33–49 μm long and 24–37 μm wide (n = 9). Planozygotes increased in size over time and eventually became brownish. Larger, darker planozygotes swam slowly, sometimes rotating on the same spot, and finally developed into walled cysts (see below).

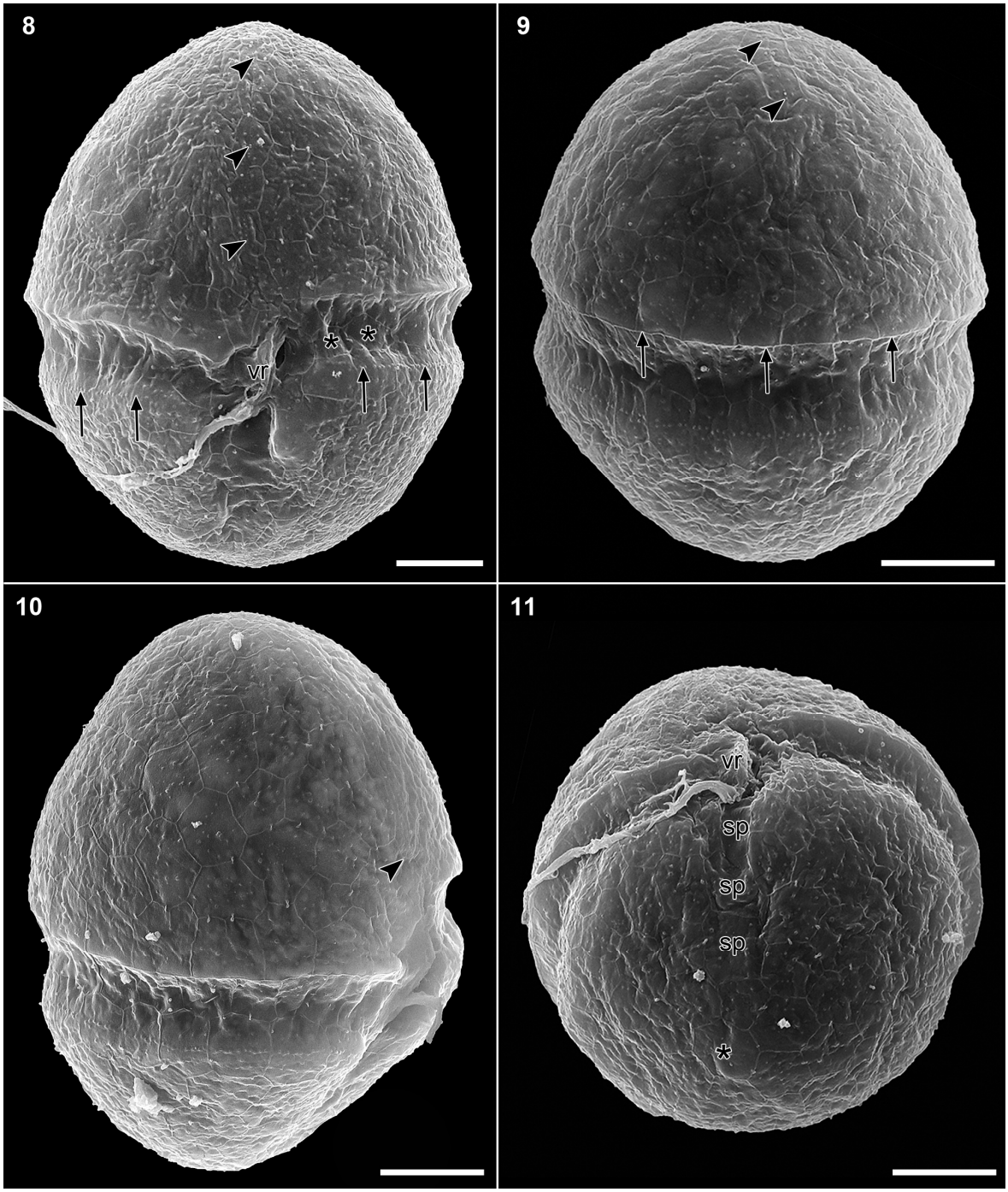
Structure of the amphiesma

The cell cover was formed by many amphiesmal vesicles containing thin thecal plates, mainly pentagonal or hexagonal, roughly arranged in latitudinal series, 5–7 on the epicone and 3–5 on the hypocone (Figs 8–12). The strict latitudinal arrangement was disrupted by occasional plates intercalated between series. In the precingular series, plates were mainly pentagonal with the posterior edges aligned to form the sharply defined anterior border of the cingulum (arrows in Fig. 9). The cingulum had two series of plates: an anterior row of pentagonal plates adjacent to the distinct upper cingulum edge, and a posterior row of mainly hexagonal plates that extended into the hypocone for about half of their length (Figs 8–10). A transverse row of knobs around the middle of the posterior row of plates surrounded the cell, marking the posterior edge of the cingulum (Figs 8–10). Most cells had five to eight knobs per plate (n = 10). Variations were observed, such as the presence of two rows of knobs in some plates, or knobs fused, scattered or disposed irregularly (not shown). On the left, proximal part of the cingulum, there were usually 1–2 additional, nearly hexagonal vesicles (Fig. 8, asterisks); similar vesicles more seldom appeared

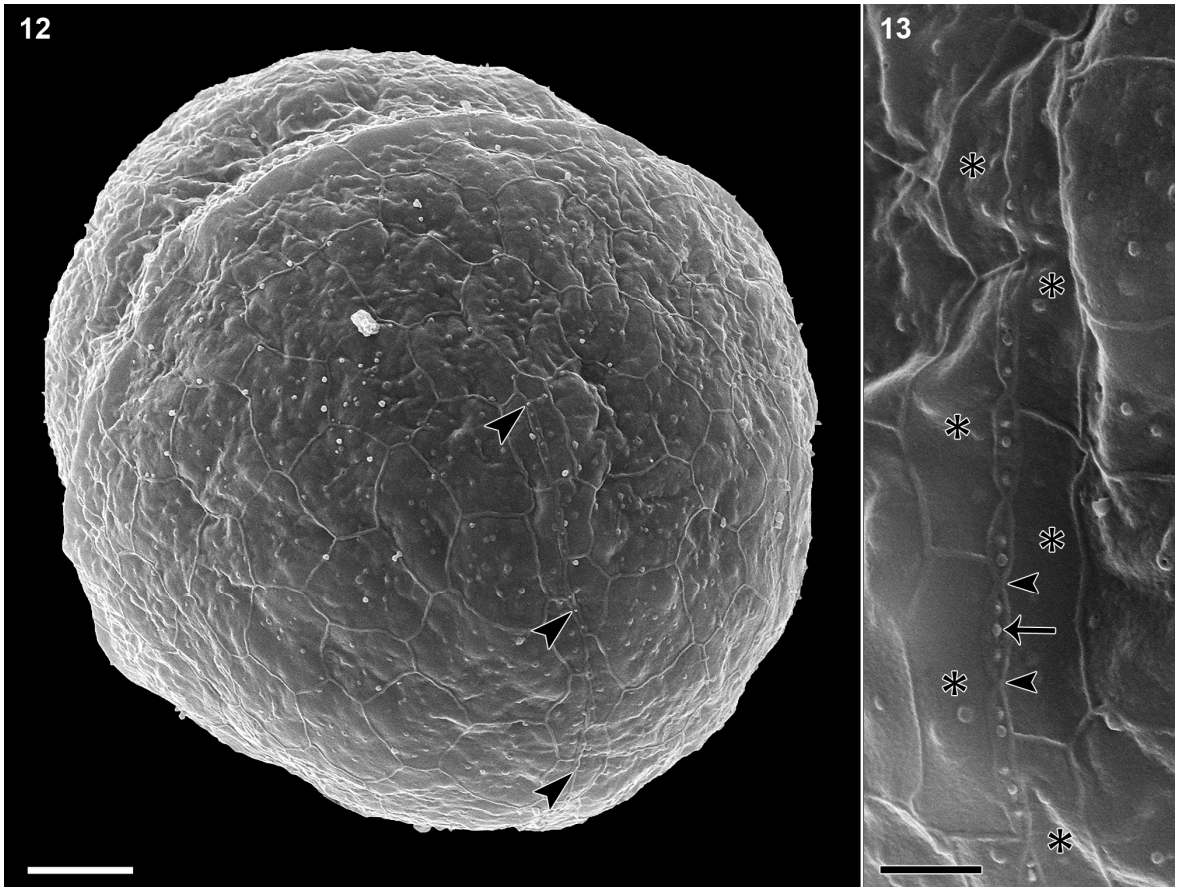
intercalated in more distal parts of the cingulum (not shown). A straight or slightly curved apical line of narrow plates (ALP) extended past the apex of the epicone, from the ventral to the dorsal side of the cell (Figs 8–10, 12, 13). The ALP was separated from the cingulum on the ventral side by one plate (Figs 8, 10) and by 3–5 plates on the dorsal side (Figs 9, 12). Plates of the ALP were 0.15–0.35 μm wide (n = 37) and 3–7 times longer, and displayed an axial row of small knobs (Fig. 13). The ALP was lined on each side by a row of narrow amphiesmal plates 0.45–1.3 μm wide (n = 30) (Figs 12, 13). Eight to nine plates were present in each of the rows of plates bording the ALP, as estimated from SEM views in which most of the structure was visible (n = 10). The antapex of the cells did not show a thickened or otherwise prominent, clearly antapical plate. A heptagonal plate is indicated in Fig. 11 (asterisk), which seems to be surrounded by regularly arranged hexagonal plates, but we did not identify a similar plate in other cells. The sulcal area was lined by 3 plates in a single row (Fig. 11). Directly anterior to the sulcus a linear elevated area above the exit pore of the cingular flagellum seemed to mark the beginning of the cingulum; on the basis of its position and appearance this was interpreted as a ventral ridge (Figs 8, 11, vr).

General internal fine structure

The general internal structure of a cell is shown in longitudinal section in Fig. 14; size and shape of this cell suggest it was a planozygote. Chloroplast lobes radiated from a central pyrenoid complex toward the periphery (Fig. 14). The deeply lobed pyrenoid area was crossed by scattered thylakoid lamellae (Fig. 15). The nucleus and a nucleolus are visible in the hypocone in Fig. 14. The pusular system was located centrally, at cingulum level (Fig. 14, arrowheads); it was formed by a tube some 250 nm wide with numerous diverticula about 160 nm long and constricted at the base (Fig. 16). An eyespot was located underneath the sulcus, beneath the longitudinal microtubular root (LMR, r1 *sensu* Moestrup, 2000), with the appearance of fused oil droplets not surrounded by a membrane (eyespot type C *sensu* Moestrup & Daugbjerg, 2007) (Fig. 17). Oil droplets (Fig. 14, o) were more prominent in the anterior cytoplasm and starch grains were present in groups between chloroplast lobes (Fig. 14, s). Scattered trichocysts were visible in the peripheral cytoplasm (Fig. 14, t).



Figs 8–11. *Tovellia aveirensis*, motile cells, SEM. 8. Ventral view. The ALP is indicated by arrowheads. The vertical arrows point to the row of knobs at the posterior edge of the cingulum. Two additional vesicles intercalated between the two regular series of cingular plates are marked at the proximal end of the cingulum (asterisks). Note the ventral ridge (vr) and the longitudinal flagellum. 9. Dorsal view. The ALP (arrowheads) is separated from the cingulum by about five series of plates. The sharply delineated anterior edge of the cingulum is marked by arrows. 10. View from the right-ventral side showing the slanting ventral face of the hypocone. The arrowhead indicates the proximal end of the ALP. 11. Aproximate antapical view showing sulcal plates (sp) arranged in a single line. The asterisk marks the antapex, where a slightly larger plate appears surrounded by latitudinal series of hypocone plates. The ventral ridge (vr) is seen raising above the cingulum-sulcus area. All scale bars = 5 μm.

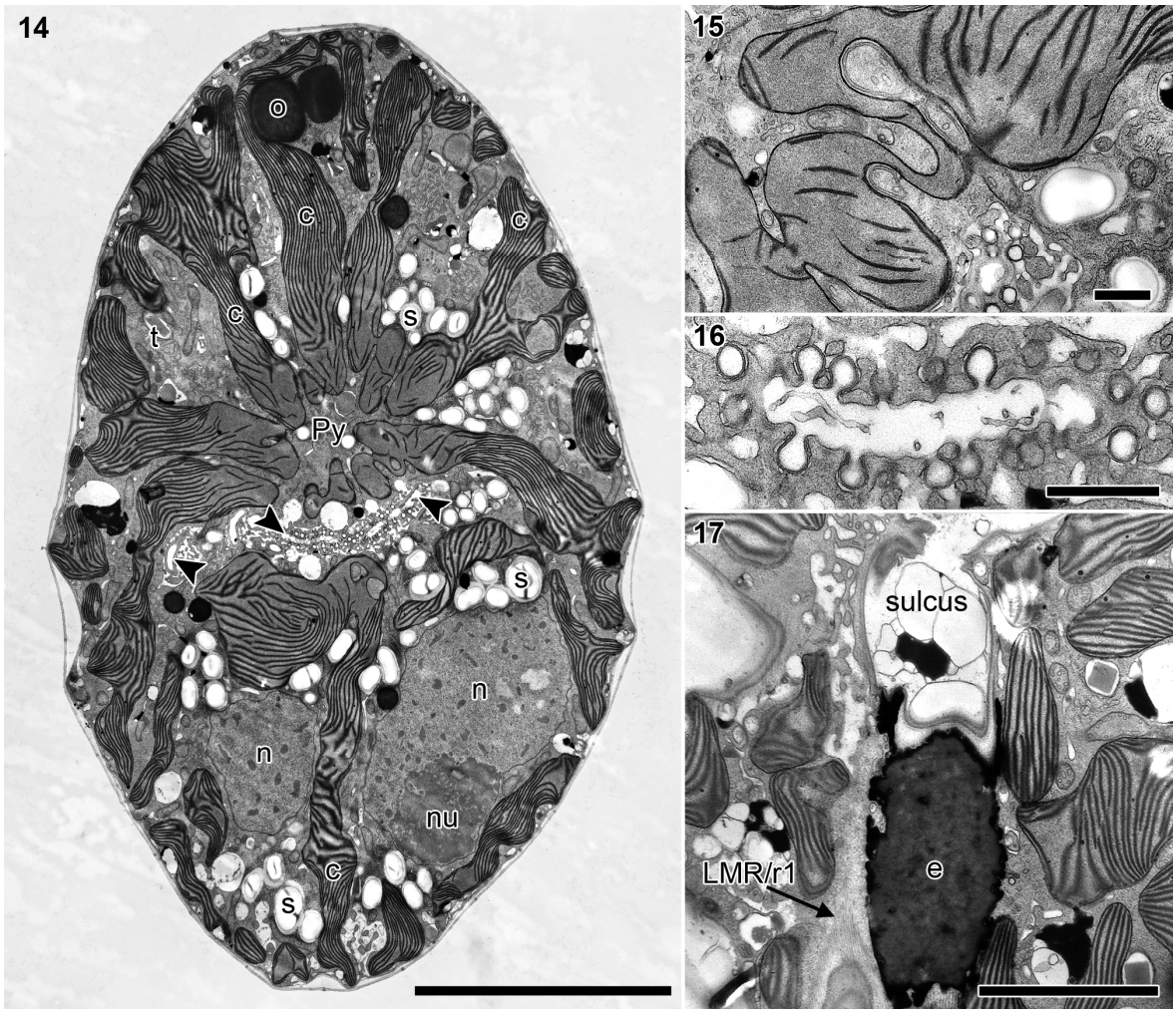


Figs 12, 13. *Tovellia aveirensis*, motile cell apex, SEM. 12. The ALP (arrowheads) extends over the epicone and is separated from the cingulum by three series of plates. 13. Detail of the ALP. Sutures between individual plates of the ALP are indicated by arrowheads. A row of small knobs (arrow) ornaments each plate of the ALP. The narrow thecal plates bordering the ALP are marked with asterisks. Scale bar in Fig. 12 = 3 μm, in Fig. 13 = 1 μm.

Cysts

Different stages of cyst development are shown in Figs 18–21. Mature cysts were elongate-ellipsoid and usually displayed a transverse constriction a little off the middle, in a position correspondent to the cingulum of planozygotes, here interpreted as a paracingulum (Figs 20, 21, 22, 24). Cyst length varied between 33 and 44 μm and width between 21 and 28 μm (n = 32). Cyst contents were usually yellowish-brown with green and colourless areas. Traces of the eyespot were sometimes visible in cysts (Fig. 20, arrow), and brown accumulation bodies were often present (Fig. 21, arrowheads). The cyst wall had a rough surface and no traces of amphiesmal plates (Figs 22–24). Mature cysts were ornamented by tapering wall processes, which were usually absent from the paracingulum area (20, 21, 22–24). Most processes were branched near the tip in a way

reminiscent of the antlers of a deer (Fig. 25). Full grown processes were 7.5–10 µm long (n = 41). Cysts in earlier stages of development were ovoid and often did not show a paracingulum (Figs 18, 19). Initial stages showed only with small protuberances (Fig. 18) whereas intermediate stages were ornamented by short, but already branched processes (Fig. 19).



Figs 14–17. *Tovellia aveirensis*, TEM of motile cell. 14. Longitudinal section through the middle of the cell, showing chloroplast lobes (c) radiating from central pyrenoid complex (Py). The somewhat curved nucleus (n) is visible in the hypocone (nu, nucleolus). A few trichocysts (t) and oil droplets (o) are visible in the peripheral cytoplasm. Starch grains (s) accumulate between chloroplasts lobes and near the antapex. Arrowheads indicate a pusular tube in the central cytoplasm. 15. Detail of central pyrenoid complex showing scattered thylakoid lamellae. 16. Pusular tube with diverticula. 17. Sulcal area in approximately grazing section, with the eyespot (e) and the longitudinal microtubular root (LMR/r1). Scale bars: Fig. 14 = 10 µm; Figs 15, 16 = 0.5 µm; Fig. 17 = 2 µm.

Asexual reproduction

Asexual reproduction occurred in the immobile stage, usually giving origin to four cells. Cells about to divide stopped on the bottom of culture wells, lost the flagella, increased in size and became more round. Cleavage furrows eventually became visible in the peripheral cytoplasm. An advanced division stage is shown in Fig. 26, with four cells already formed inside the division cyst. The daughter cells in this stage appeared already formed, with visible furrows and outward-facing eyespots, but no traces of flagella. Segregation and subsequent release of daughter cells usually occurred over a few minutes, during which cells slowly slid apart and emerged through an opening in the somewhat inflated cover of the division cyst (Figs 27, 28). Shortly after, flagella became visible and cells started to swim, one after another (Fig. 28, 29). Division cysts with only two cells were also seen, but daughter cell release from those cysts was not observed.

Molecular phylogeny

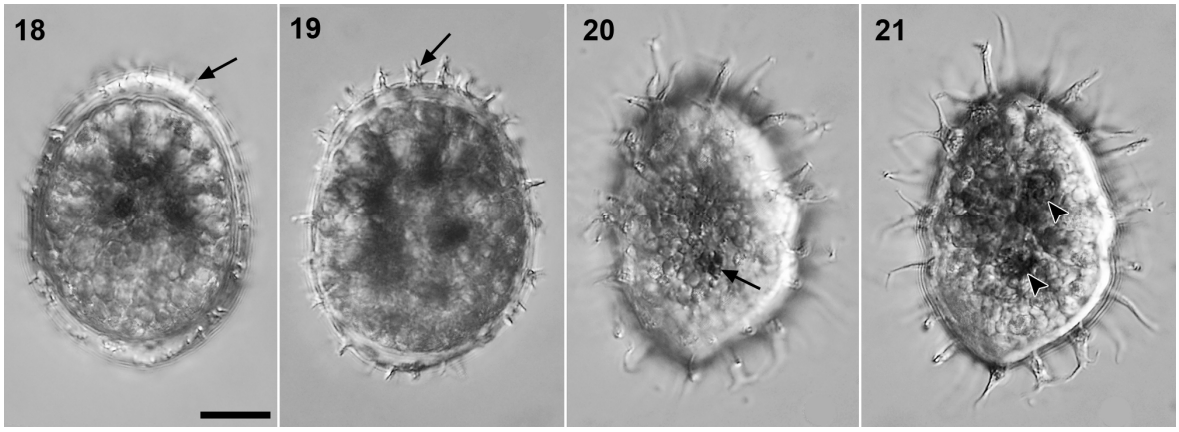
The phylogenetic relationships of *Tovellia aveirensis* are shown in Fig. 30. *Tovellia* formed a monophyletic genus highly supported by Bayesian (posterior probability, pp = 1.0) and maximum likelihood analyses (bootstrap support, BS = 100%). *Tovellia aveirensis* formed a sister taxon to *T. sanguinea*. However, this relationship received high bayesian support (pp = 0.97) but only moderate bootstrap support (67.6%). *Tovellia coronata* formed a sister taxon to the *T. sanguinea*/*T. aveirensis* clade. The family Tovelliaceae received high support from Bayesian analysis (pp = 1.0) but only 70.3% in ML bootstrap support. Within Tovelliaceae *Esoptrodinum gemma* formed a sister taxon to *Tovellia*, and *Jadwigia* a sister taxon to *Tovellia* spp. and *E. gemma* (Fig. 30). Except for the early branching of *Moestrupia oblonga* (pp = 0.85 and BS < 50%) the topology of the deepest lineages was unresolved in the phylogenetic analyses conducted here (Fig. 30). However, the class Dinophyceae received high support (pp = 1.0, BS = 99.8%).

Sequence divergence estimates were based on 1027 base pairs, including introduced gaps (Table 1). Despite the supposedly close relationship between *Tovellia* spp., *Esoptrodinium gemma* and *Jadwigia applanata* (all members of the family Tovelliaceae) the highly variable domain D2 could not be aligned unambiguously. Hence, it was excluded prior to sequence divergence estimations. As expected the highest sequence divergences were seen in all pairwise comparisons between *Jadwigia*/*Esotrodinium* and

any of three *Tovellia* species (ranging from 12.4–16.5% based on p-values and 13.7–18.8% based on Kimura-2-parameter values). The lowest divergence estimate was seen when comparing *Tovellia coronata* and *T. aveirensis* (≈ 4% in both calculations). The divergence between *T. sanguinea* and *T. aveirensis* was 6.3% or 6.7% and between *T. sanguinea* and *T. coronata* it was 5.1% or 5.3%, depending on the calculation method (p-values versus K-2-p model) (Table 1).

Table 1. Sequence divergences (in percentage) of three species of *Tovellia*, *Esoptrodinium gemma* and *Jadwigia applanata* based on 1027 LSU rDNA nucleotides. Uncorrected distances (p-values in PAUP*) are given above the diagonal, and distance values calculated using Kimura-2-parameter model are given below the diagonal.

	Tovellia aveirensis	***Tovellia sanguinea***	***Tovellia coronata***	***Esoptrodinium gemma***	***Jadwigia applanata***
T. aveirensis	–	6.3	4.0	16.5	14.5
T. sanguinea	6.7	–	5.1	16.5	14.4
T. coronata	4.1	5.3	–	15.2	13.2
E. gemma	18.8	18.8	17.2	–	12.4
J. applanata	16.4	16.2	14.8	13.7	–



Figs 18–21. *Tovellia aveirensis*, cysts, LM. 18, 19. Cysts in early stages of development, ovoid in shape and without a marked paracingulum. Small, unbranched protuberances are visible in Fig. 18, while distal branches are already visible in Fig. 19 (arrows). 20, 21. Surface and deeper focus of a mature cyst, showing a middle equatorial constriction (paracingulum). The wall is ornamented by long, branched processes. The arrow in Fig. 20 indicates the remainder of the eyespot. Accumulation bodies are visible in Fig. 21 (arrowheads). All Figs at the same scale. Scale bar = 10 μm.

DISCUSSION

Morphology and taxonomic affinities

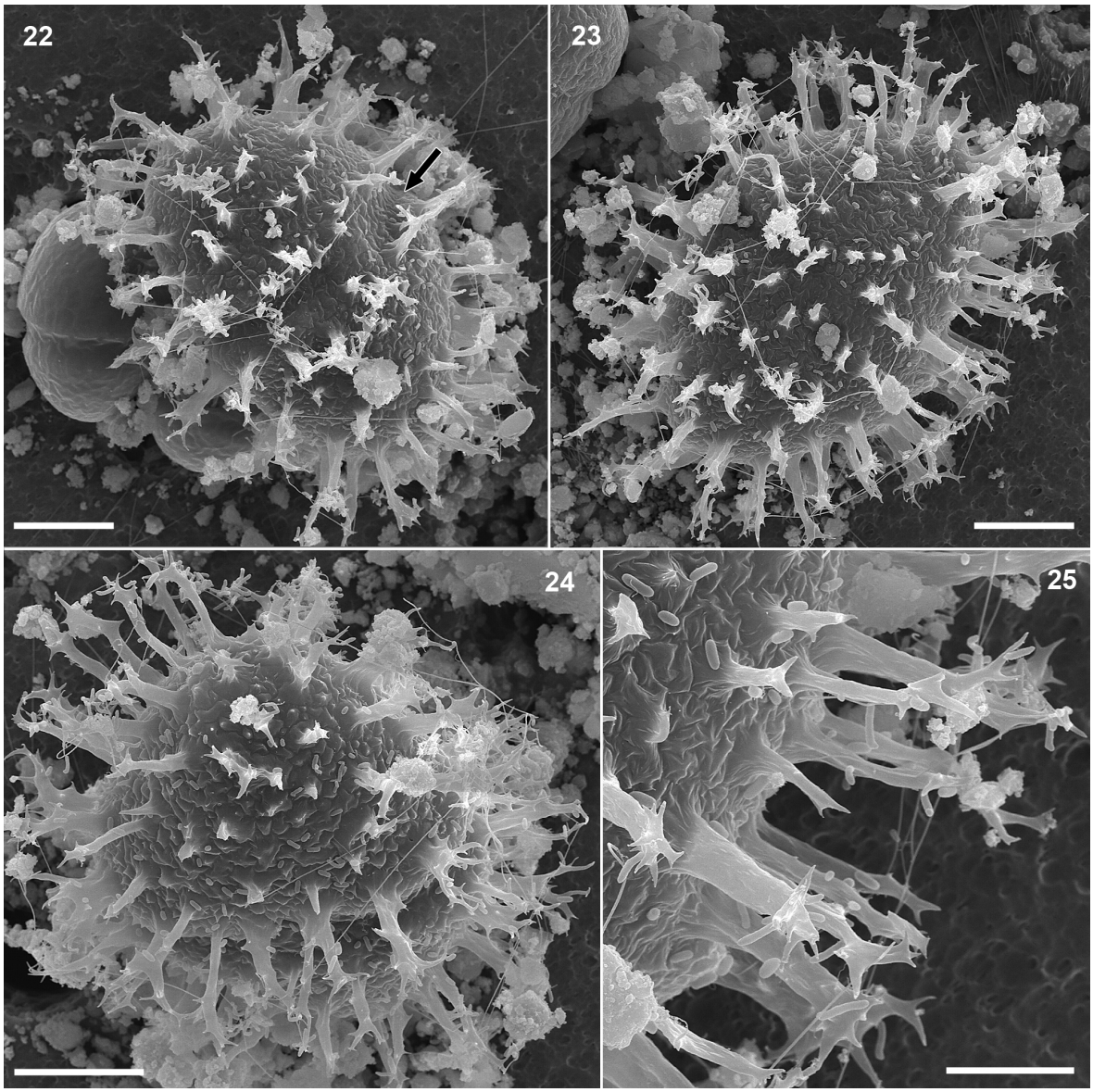
The tovelliacean affinity of live swimming cells of the species we report on is mainly suggested by the prominent, trough-shaped eyespot combined with the firm, thin cell cover that gives them a woloszynskioid appearance. The confirmation of the type C eyespot (Moestrup & Daugbjerg, 2007) and the demonstration of a tubular pusule with diverticula, both by TEM observations, firmly place the new species in the Tovelliaceae (see summary of fine-structural tovelliacean characters in Calado, 2011). Of the four genera currently classified in the Tovelliaceae, both *Esoptrodinium/Bernardinium* and *Opisthoaulax* show a strongly asymmetric external morphology, quite different from the organism described here (Javornický, 1997; Calado *et al.*, 2006; Calado, 2011). The motile cells of the two other tovelliacean genera, *Tovellia* and *Jadwigia*, differ essentially in that *Tovellia* has two rows of narrow plates bordering the ALP whereas in *Jadwigia* the ALP is in contact with plates that are not fundamentally different from other amphiesmal plates on the epicone (Lindberg *et al.*, 2005). The two rows of narrow plates lining the ALP of the species described herein clearly point to *Tovellia*.

Another link to *Tovellia* is the morphology of the resting cysts produced in cultures of *T. aveirensis*. The different cyst types produced by woloszynskioids were among the first pieces of evidence suggesting the group was polyphyletic (Stosch, 1973), and cyst morphologies have been used as generic level characters in the group (Lindberg *et al.*, 2005; Moestrup & Daugbjerg, 2007). Wolozynskioid resting cysts that are clearly bipolar and show an equatorial constriction or paracingulum have only been found in *Tovellia*, in *Opisthoaulax* and in the marine suessiacean species *Polarella glacialis* Montresor, Procaccini & Stoecker (Montresor *et al.*, 1999; Moestrup *et al.*, 2009*a*; Calado, 2011). Vegetative cell characters and habitat separate *Opisthoaulax* and *Polarella* from *T. aveirensis*.

Comparison with other *Tovellia* species

Seven species are currently classified in the genus *Tovellia*. The strongly flattened motile cells of *T. leopoliensis* (Wołoszyńska) Moestrup, K. Lindberg & Daugbjerg, and the sharply pointed or projecting apices of both this species and *T. apiculata* (Stosch)

Moestrup, K. Lindberg & Daugbjerg contrast with the overall round appearance of *T. aveirensis* swarmers (Wołoszyńska, 1917; Stosch, 1973). Swimming cells and cysts of both *T. coronata* (Wołoszyńska) Moestrup, K. Lindberg & Daugbjerg and *T. sanguinea* Moestrup, Gert Hansen, Daugbjerg, Flaim & d'Andrea typically display cytoplasmic accumulations of red pigment, sometimes to the point of large populations of these species conferring a strong red colour to the water (Flaim *et al.*, 2004; Lindberg *et al.*, 2005; Moestrup *et al.*, 2006). Neither swimming cells nor resting cysts of *T. aveirensis* were ever



Figs 22–25. *Tovellia aveirensis*, mature cysts, SEM. 22, 23. Lateral views of cysts showing the long, tapering spines. The middle constriction is marked with an arrow in Fig. 22; it is not distinct in Fig. 23. Cyst surface is rough, without traces of amphiesmal plates. Note smaller swarmer of *T. aveirensis* to the left of the cyst in Fig. 22. 24. Polar view of cyst. 25. Detail of the branched spine tips. Scale bars in Figs 22–24 = 10 μm; in Fig. 25 = 5 μm.

seen to acquire a red colour regardless of the age or state of growth of the batch cultures examined. The little known *T. glabra* (Wołoszyńska) Moestrup, K. Lindberg & Daugbjerg was originally described as a variety of *T. coronata* from which it differed only in not having a prominent, punctate antapical plate (Wołoszyńska, 1917); Thompson (1951) described *T. coronata*-like cells without such a plate, therefore identifiable as *T. glabra*, without noting red cytoplasmic bodies. Thompson's (1951) illustrations of *T. glabra* show cells shorter and more broadly rounded epicones than in *T. aveirensis*, and Wołoszyńska's (1917) concept of the taxon included larger amphiesmal plates, distributed over a smaller number of latitudinal rows, than shown here for *T. aveirensis*.

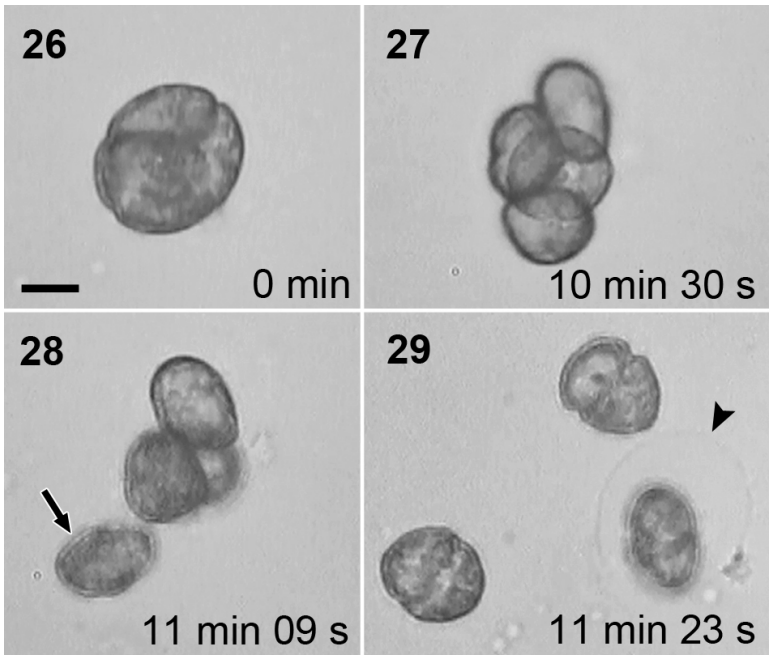
Tovellia nygardii Moestrup, K. Lindberg & Daugbjerg was described as a somewhat smaller species, with amphiesmal plates distinctly larger and fewer than in *T. aveirensis*, notably with only one row a large plates in the cingulum (Christen, 1958; Moestrup *et al.*, 2008). *Tovellia stoschii* (Shyam & Sarma) Moestrup, K. Lindberg & Daugbjerg has a large number of relatively small plates arranged in about 17 latitudinal rows and its conical epicone is distinctly smaller than the hypocone (Shyam & Sarma, 1976).

Cysts were described for five *Tovellia* species, all with horns extending from the apex and antapex, and a constriction or paracingulum in the middle, sometimes with pre- and post-cingular rows of roundish protuberances, in other cases with staight or somewhat conical, non-ramified spines (Stosch, 1973; Wołoszyńska, 1917; Christen, 1958; Moestrup *et al.*, 2006). With its distally ramified, tapering spines and the absence of prominent apical and antapical projections the cyst of *T. aveirensis* is quite distinct from any of those previously described.

Reliable identification of *Tovellia* species from observations on motile cells remains a demanding task that usually requires detailed observation of the cell cover. This is nearly impossible to accomplish in live cells, and even with SEM observations of adequately prepared material uncertainty may arise from individual variation and from the lack of comparative SEM studies on several of the species. A constant feature of cells of *T. aveirensis* observed in SEM was a line of knobs across the posterior row of cingular plates that seemed to mark the posterior edge of the cingulum. This has not been demonstrated in other species of *Tovellia* and is therefore suggested as an additional marker of the identity of *T. aveirensis*.

Asexual reproduction

Asexual reproduction has not been described for all species presently included in the genus *Tovellia*. However, all available reports of this process indicate that it occurs through the division of non-motile cells, usually into two to eight zoospores (Wołoszyńska, 1917; Christen, 1958; Stosch, 1973; Shyam & Sarma, 1976). Asexual reproduction of *T. aveirensis* is generally similar to that described for other *Tovellia* species, in particular to *T. apiculata* (Stosch, 1973) and *T. stoschii* (Shyam & Sarma, 1976). In all cases where successful release of daughter cells from division cysts of *T. aveirensis* was observed, the number of cells was four. Observations of division cysts containing only two cells was never followed by the release of those cells, and neither did we observe division cysts with a number of cells higher than four. This suggests that, under the culture conditions adopted, division cysts of *T. aveirensis* produce mainly four offspring cells and that a second round of cell division was likely to occur in the cysts with only two cells.



Figs 26–29. *Tovellia aveirensis*, asexual reproduction, LM. The images were prepared from still frames of a video recording and are marked with the time elapsed between the moments they were recorded. 26. Division cyst with four daughter cells already formed inside. 27. Beginning of cell separation. The four cells started to slide apart and emerging from the division cyst (the cyst cover is marked with an arrowhead in Fig. 29). Cells did not have developed flagella at this stage. 28. The first cell swam away from the group (arrow). The flagella of the remaining cells also begin to be visible in the video recording at this point (although they are indistinct in the still image). 29. Only one cell still remains within in the division cyst cover (arrowhead); it swam away seconds later. All Figs at the same scale. Scale bar = 10 μm.

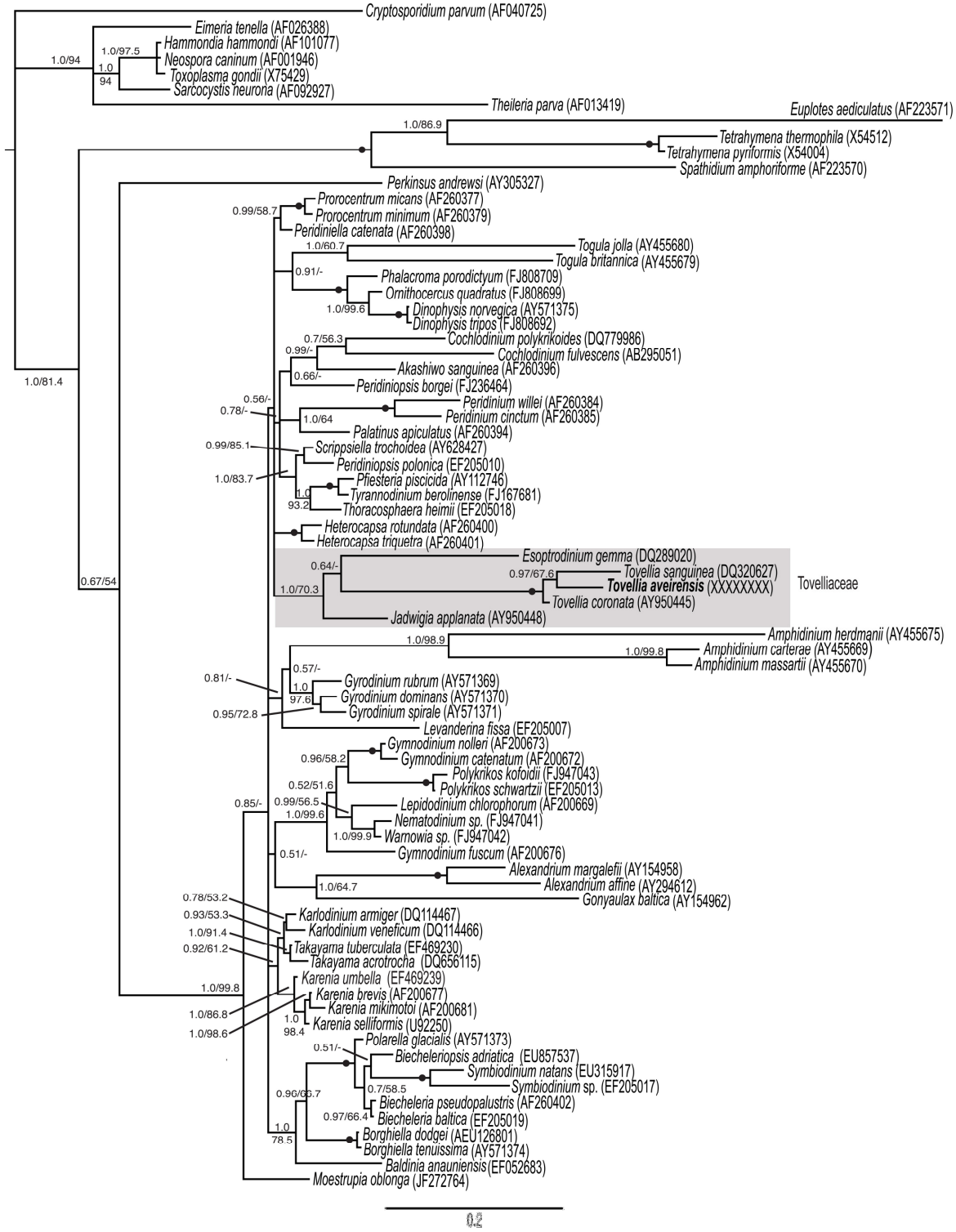


Fig. 30. Molecular phylogeny of *Tovellia aveirensis* sp. nov. (in bold face) and 63 other dinoflagellate species inferred from Bayesian analysis of nuclear-encoded LSU rDNA sequences. Four ciliates, seven apicomplexans and *Perkinsus* formed the outgroup. The first numbers at internal nodes are posterior probabilities (≥0.5) from bayesian analysis (BA) and the last numbers are bootstrap values (≥ 50%) from maximum likelihood (ML) with 1,000 replications. Filled circles illustrate the highest possible support in BA and ML (1.0 and 100%, respectively). GenBank accession numbers in parentheses. The family Tovelliaceae is marked in grey.

Phylogeny

In spite of a smaller sequence divergence between *T. aveirensis* and *T. coronata* than between *T. aveirensis* and *T. sanguinea* the phylogenetic analysis grouped the latter two species in a poorly supported clade. Both in the arrangement of ampiesmal plates, including the organization of the ALP, and in the tendency for accumulating red cytoplasmic bodies the species *T. coronata* and *T. sanguinea* (Lindberg *et al.*, 2005; Moestrup *et al.*, 2006) seem more closely related than either to *T. aveirensis*. One structural aspect in which the new species approaches *T. sanguinea* is the radial arrangement of chloroplast lobes in the epicone, converging to a central pyrenoid complex, which is reportedly absent in *T. coronata* (Lindberg *et al.*, 2005; Moestrup *et al.*, 2006). However, the limited number of species for which LSU rDNA sequences are available renders any conclusions about the close affinities between species of *Tovellia* premature.

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CHAPTER 4

STUDIES ON WOLOSZYNSKIOID DINOFLAGELLATES VII. DESCRIPTION OF *BORGHIELLA ANDERSENII* SP. NOV.: LIGHT AND ELECTRON MICROSCOPY AND PHYLOGENY BASED ON LSU RDNA

An article with the description of *B. andersenii* sp. nov. is being prepared in collaboration with researchers of the University of Copenhagen, Denmark, which have been working with specimens collected in Scotland. These are morphologically identical to those found in Portugal, and a comparison of their partial LSU rDNA sequences has confirmed them as members of the same species. In this chapter will be presented and discussed the results obtained from the Portuguese specimens, with consideration of the results of the Danish coworkers whenever they allow complementing or improving the species description. The planned title for the article in preparation is given below.

Daugbjerg, N., Andreasen, T., Happel, E., Pandeirada, M.S., Hansen, G., Craveiro, S.C., Calado, A.J. & Moestrup, Ø.. Studies on woloszynskioid dinoflagellates VI. Description of *Borghiella andersenii* sp. nov.: light and electron microscopy and phylogeny based on LSU rDNA. *European Journal of Phycology* (in preparation) – Inclusion in this thesis is not intended as effective publication of the new taxon proposed in the manuscript

ABSTRACT

The Borghiellaceae constitutes a family of dinoflagellates that assembles freshwater woloszynskioids of the genera *Borghiella* and *Baldinia*, with a Moestrup & Daugbjerg's (2007) eyespot type B. Here is described a new *Borghiella* species, *B. andersenii* sp. ined., collected in freshwaters of Portugal and Scotland. Morphologically, it is very close to *Borghiella dodgei*, diverging from it mainly by having a rounded epicone and a shorter pair of elongate amphiesmal vesicles (PEV) with fewer knobs and lined on each side by two to three apical plates. A phylogenetic hypothesis based on partial large subunit ribosomal RNA gene sequences also supports the description of the new species. Asexual reproduction in non-motile stage involving the formation of division cysts is here described for the first time for the family Borghiellaceae. Stronger evidences of sexual reproduction, namely planozygotes and apparent resting cysts, are also presented.

Key words: asexual and sexual reproduction, *Borghiella,* dinoflagellates, Dinophyceae, LSU rDNA, phylogeny, ultrastructure

INTRODUCTION

Borghiellaceae, Tovelliaceae and Suessiaceae are the three dinoflagellate families that have received most of the wolosynskioid dinoflagellates after the recognition that they comprised a polyphyletic assemblage (Lindberg *et al.,* 2005; Moestrup *et al.,* 2008; Moestrup *et al.,* 2009). Borghiellaceae, in particular, holds the freshwater woloszynskioids from the recent genera *Borghiella* Moestrup, Gert Hansen & Daugbjerg and *Baldinia* Gert Hansen & Daugbjerg, with an eyespot made of osmiophilic globules located in a chloroplast lobe and one layer of brick-like units between the chloroplast lobe and the sulcal amphiesmal plates (type B in Moestrup & Daugbjerg, 2007; Moestrup *et al.,* 2008; Moestrup *et al.,* 2009). *Borghiella* species are covered by many, thin amphiesmal plates, usually hexagonal, and have an apical complex constituted by one pair of elongate amphiesmal vesicles (PEV), which are exclusive of the genus (Moestrup *et al.,* 2008). In contrast, *Baldinia anauniensis* Gert Hansen & Daugbjerg lacks both thecal plates and an apical complex (Hansen *et al.,* 2007).

As for most woloszynskioids, the life cycle of members of Borghiellaceae is poorly understood. There are only, to our knowledge, references to asexual reproduction in the motile stage, by fission, in *Borghiella dodgei* Moestrup, Gert Hansen & Daugbjerg (Moestrup *et al.,* 2008) and *Borghiella tenuissima* (Lauterborn) Moestrup, Gert Hansen & Daugbjerg (Stosch, 1973), and of sexual reproduction based on the observation of apparent resting cysts, which are thought to be formed following gamete fusion, as indicated for *Baldinia anauniensis* (Hansen et *al.,* 2007) and *Borghiella tenuissima* (Stosch, 1973).

The present work describes a new species of *Borghiella* collected from freshwater in Portugal and Scotland. Morphologically, it is very similar to the previously described *B. dodgei*, diverging from it mainly by having a rounded epicone and a shorter PEV, with fewer knobs and lined on each side by two to three apical plates. Asexual reproduction in non-motile stages with production of division cysts and strong evidence of sexual reproduction are also presented. A LSU rDNA-based phylogeny supports the taxonomic placement of the new species.

MATERIAL AND METHODS

Biological material

The species here described was found in a net sample (mesh size 25-30 μm) collected in a flooded area known as Ribeiro da Palha, located nearby the village of Nariz, Aveiro, Portugal, on 22 November 2010. Four swimming cells were transferred to one culture well with L16 medium (Lindström, 1991) supplemented with vitamins according to Popovský & Pfiester (1990) and one swimming cell to another well filled with the same medium. The cells in both wells were maintained at 18ºC with 12:12 light:dark photoperiod. Two culture lines were thus initiated, named MSP2 and MSP8.

Light microscopy (LM)

Light micrographs of vegetative cells, planozygotes, non-motile asexual stages and cysts were taken with a Zeiss Axioplan 2 imaging light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a DP70 and a ColorView IIIu Olympus cameras (Olympus Corp., Tokyo, Japan).

Asexual reproduction in the motile stage was recorded with a JVC TK-C1481BEG color video camera (Norbain SD Ltd, Reading, United Kingdom) mounted on a Leitz

Biomed light microscope and on a Leitz Labovert FS inverted light microscope (Leica Microsystems, Wetzlar, Germany). Illustrations of this process were taken from still frames of the recorded videos.

Scanning electron microscopy (SEM)

Vegetative cells were prepared according to the following procedure: 1 ml of culture was fixed, for 15 minutes, by addition of 1 ml of a fixative mixture made of saturated $HgCl_2$ and 2% osmium tetroxide in a proportion of 1:5. After fixation, cells were retained on 8-µm pore size Isopore polycarbonate filters (Millipore Corp., Billerica, USA) and washed in distilled water for about 45 min. Dehydration of filters with the cells was performed with a graded ethanol series, with a stop at 70% ethanol overnight, at 4ºC. Dehydrated material was critical point dried. The dried filters were glued onto stubs, sputter-coated with gold-palladium and examined with a Hitachi S-4100 (Hitachi High-Technologies Corp., Tokyo, Japan) scanning electron microscope (SEM).

Single cell PCR amplification of LSU rDNA

Cells of MSP2 and MSP8 culture lines were used for PCR amplification. One to four swimming cells were isolated with a micropipette under the inverted microscope, transferred to 0.2-ml PCR tubes and frozen at -8ºC for 3 or 4 days, before the PCR reactions. Cell DNA was the template to amplify about 1500 base pairs (bp) of the LSU rRNA gene using the terminal primers D1R (Scholin *et al.,* 1994) and 28-1483R (Daugbjerg *et al.,* 2000). These were added to the PCR tubes with the isolated cells, followed by the beads containing all chemicals necessary to the PCR amplification (illustra^{TM} puReTaq Ready-To-Go PCR Beads (GE Healthcare UK Ltd)). The reaction took place in the Biometra–Tprofessional thermocycler (Biometra GmbH, Goettingen, Germany). The thermal profile for MSP2 included one initial cycle of denaturation at 94ºC for 3 min; 35 cycles of each of the following steps: denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min, and extension at 72ºC for 3 min; and the final cycle of extension at 72ºC for 10 min. The thermal profile for MSP8 is outlined in Moestrup *et al.* (2008), from which it differs only in using a longer final cycle of extension, 10 min instead of 6 min. The DNA fragments were loaded onto a 1% agarose gel, run for 20 min at 90 V and viewed under a UV light table (Molecular imager chemiDoc XRS System, Bio-Rad

Laboratories, Inc., Hercules, California, USA). As the PCR bands of MSP2 were weak, another PCR amplification was performed using the PCR product of the first attempt (nested-PCR). A volume of 2 µl of product from the first PCR was transferred to two 0.2-ml PCR tubes containing illustra^{TM} puReTaq Ready-To-Go PCR Beads. To one tube we added 1.25 µl of the primers D1R and D3A (Hansen *et al.,* 2000) and to the second tube we added 1.25 µl of the primers D3B (Hansen *et al.,* 2000) and 28-1483R. The thermal profile of the nested-PCR included the initial denaturation at 94ºC for 3 min and 18 cycles of each of the following steps: denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min, and extension at 72ºC for 3 min; and the final cycle of extension at 72ºC for 6 min. PCR and nested-PCR products, of MSP8 and MSP2 culture lines respectively, were purified using the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's recommendations, and sent to Macrogen Europe (Amsterdam, The Netherlands) for sequence determination in both directions. The sequencing primers used were D1R, D2C, D3A, D3B and 28-1483 (Hansen *et al.,* 2000).

Alignment and phylogenetic analyses

The editing of partial LSU rDNA sequences of cells of MSP2 and MSP8 culture lines as well as their alignment with 16 dinoflagellate sequences obtained from GenBank (Table 1) and 5 woloszynskioid dinoflagellates sequences from our culture collection was performed using the Geneious plataform (ver. 6.0.3, Biomatters Ltd. Auckland, New Zealand). The thecate dinoflagellate *Peridinium cinctum* (O.F.Müller) Ehrenberg was used to root the ingroup of the other dinoflagellates, constituting therefore the outgroup. The data matrix consisted of 1046 bp, including introduced gaps and without the highly divergent D2 domain. A Bayesian analysis (BA) was performed in the Geneious plataform, using MrBayes with the following parameters (Huelsenbeck & Ronquist, 2001): HKY85 substituion model; rate variation = gamma; gamma categories = 4. The Metropolis-coupled Monte Carlo Markov chains (MCMC) was used to estimate posterior probabilities. This analysis (MCMC) was running with four heated chains for 1.1×10^6 generations at a temperature of 0.2 and subsampling frequency of 200 with a burn-in length of 10^5. The priors were unconstrained branch lenght, exponential (10). The tree was visualized with Geneious and the Bayesian posterior probabilities registered in the nodes.

Table 1. List of dinoflagellates and GenBank accession numbers of partial LSU rDNA sequences used in the phylogenetic analysis

Species	Genbank accession numbers
Baldinia anauniensis Gert Hansen & Daugbjerg	EF052683
Bernardinium bernardinense Chodat	DQ289020
Biecheleria baltica Moestrup, K. Lindberg & Daugbjerg	EF205019
B. pseudopalustris (J. Schiller) Moestrup, K. Lindberg & Daugbjerg	AF260402
Biecheleriopsis adriatica Moestrup, K. Lindberg & Daugbjerg	EU857537
Borghiella dodgei Moestrup, Gert Hansen & Daugbjerg	EU126801
B. tenuissima (Lauterborn) Moestrup, Gert Hansen & Daugbjerg	AY571374
Jadwigia applanata K. Lindberg, Moestrup & Daugbjerg	AY950448
Peridinium cinctum Ehrenberg	EF205011
Polarella glacialis Montresor, Procaccini & Stoecker	AY571373
Protodinium simplex Lohmann	EF205014
Sphaerodinium cracoviense Wołoszyńska	HQ176319
Symbiodinium natans Gert Hansen & Daugbjerg	EU315917
Symbiodinium sp.	EF205017
Tovellia coronata K. Lindberg, Moestrup & Daugbjerg	AY950445
T. sanguinea Moestrup, Gert Hansen, Daugbjerg, G. Flaim & D'Andrea	DQ320627

RESULTS

Borghiella andersenii sp. ined. (Figs 1–18)

Description: vegetative cells spherical and somewhat compressed dorsoventrally. Cingulum displaced about one cingulum width. Epi- and hypocone rounded, similar in size or the former slightly larger. Hypocone somewhat more flattened than the epicone. Sulcus widening posteriorly into a concave area, covered in its upper part by a ventral ridge. Cells 17–24 µm long, 12–20 µm wide and 12–18 µm thick. Eyespot type B, *sensu* Moestrup & Daugbjerg (2007), not easily seen in LM. Chloroplast lobes golden to yellowish-green, disposed at the periphery of the cell. Nucleus central and extending into the epicone. Cell cover composed of amphiesmal vesicles arranged in latitudinal series, five or six on the epicone and three to five on the hypocone. A narrow pair of elongate amphiesmal vesicles (PEV), 1.5–4.5 µm long, present in the epicone. Asexual reproduction both in motile stage,

by fission, and in non-motile stage, with production of division cysts. Sexual reproduction with formation of planozygotes with two longitudinal flagella. Resting cysts with a smooth wall, spherical to elongate, with one long or several shorter reddish-brown (rarily orange) accumulation bodies.

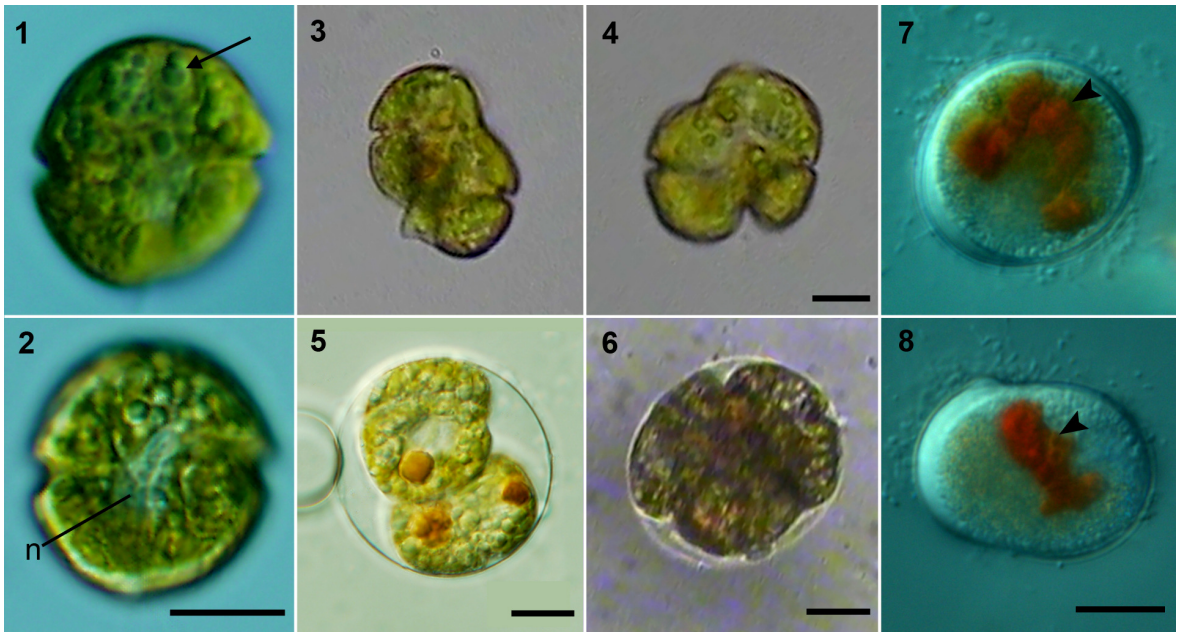
Holotype: yet to be designated taking into account material collected in Scotland.

Type locality: locality in Scotland, or Flooded area in Ribeiro da Palha stream, Nariz, Aveiro (40°33'14.42"N, 8°34'5.73"W).

Etymology: the species epithet *andersenii* honors of Robert A. Andersen, a specialist in chrysophytes *sensu lato* and in culturing freshwater and marine species, who first established a culture of this species from material collected in Scotland.

General morphology of motile cells

Motile cells are illustrated in Figs 1–4, 9–16. Vegetative cells were usually spherical and slightly compressed dorsoventrally (Figs 1, 2, 9–13). The cingulum was displaced about one cingulum width (Figs 1, 2, 9, 10). The rounded epi- and hypocone had a similar size or the epicone was slightly larger (Figs 1, 2, 9, 12). The hypocone was somewhat more flattened than the epicone (Figs 10, 13). The sulcus widened posteriorly into a concave area (Figs 9, 10, 13). Cells were 17–24 µm long, 12–20 µm wide (n = 30) and 12–18 µm thick (n = 12). The eyespot was usually not noticeable in LM (Figs 1, 2). The golden to yellowish-green chloroplast were disposed at the periphery (not shown). Several round and greenish bodies were usually present in the cytoplasm, probably corresponding to lipid reserves or starch (Fig. 1, arrow). The nucleus was located centrally and extended into the epicone (Fig. 2). Planozygotes were identified by the presence of paired longitudinal flagella (Fig. 18).



Figs 1–8. LM of *Borghiella andersenii* sp. ined. Figs 1, 2. Ventral view of vegetative cells showing the cingulum displaced about one cingulum width. n, nucleus. Several round and greenish bodies (lipid reserves or starch) marked with an arrow. Figs 3, 4. Ventral and dorsal views of swimming division pairs, respectively. Figs 5, 6. Division cysts with two and four cells inside, respectively. Figs 7, 8. Resting cysts. The arrowheads point to the red bodies. The following Figs are at the same scale: Figs 1, 2; Figs 3, 4; Figs 7, 8. All scale bars = 10 µm.

Structure of the amphiesma

The cell cover was composed of many amphiesmal vesicles containing thin thecal plates, mainly hexagonal, arranged in latitudinal series, five or six on the epicone and three to five on the hypocone (Figs 9–13). These series were sometimes interrupted by intercalary plates. The precingular series included pentagonal plates and some nearly rectangular or quadrangular variations (Figs 9–11), whose lower side was parallel to the cingulum, forming a distinctive anterior border (arrows in Fig. 15). The cingulum had three series of plates: one row at the anterior border with pentagonal and some nearly rectangular plates (Fig. 15, S1); a middle row with mostly hexagonal and some pentagonal or rhombic plates (Fig. 15, S2); and a posterior row that extended into the hypocone, with hexagonal plates and some nearly rectangular or quadrangular variations (Fig. 15, S3). In the epicone there was a straight or slightly curved, narrow pair of elongate amphiesmal vesicles (PEV *sensu* Moestrup *et al.,* 2008) (Figs 11, 14, 16). The PEV was 1.5–4.5 µm long, more often 3.2–3.8 µm (8 out of 14 measurements), and had 9–17 axial knobs. The PEV was lined on

each side by two or three apical plates, usually pentagonal or rectangular. The sulcal area was composed of about seven plates, generally hexagonal (Fig. 13). An extension of the epicone above the area where the flagella exit the cell was interpreted as the ventral ridge (Fig. 9, vr). Plate surface was more or less smooth and had few circular pores randomly distributed (perhaps trichocyst pores). The boundaries between plates were well delineated.

Asexual division stages and sexual stages

The species here described reproduced asexually both in the motile and in non-motile stages. In the former case, cells divided obliquely by fission while swimming. They were often seen in advanced stages of division, with the two daughter cells already perfectly identifiable (Figs 3, 4, 17). Near the separation moment, the cells were usually connected by their epicones, with the upper cell almost horizontally positioned over the lower one (Fig. 17).

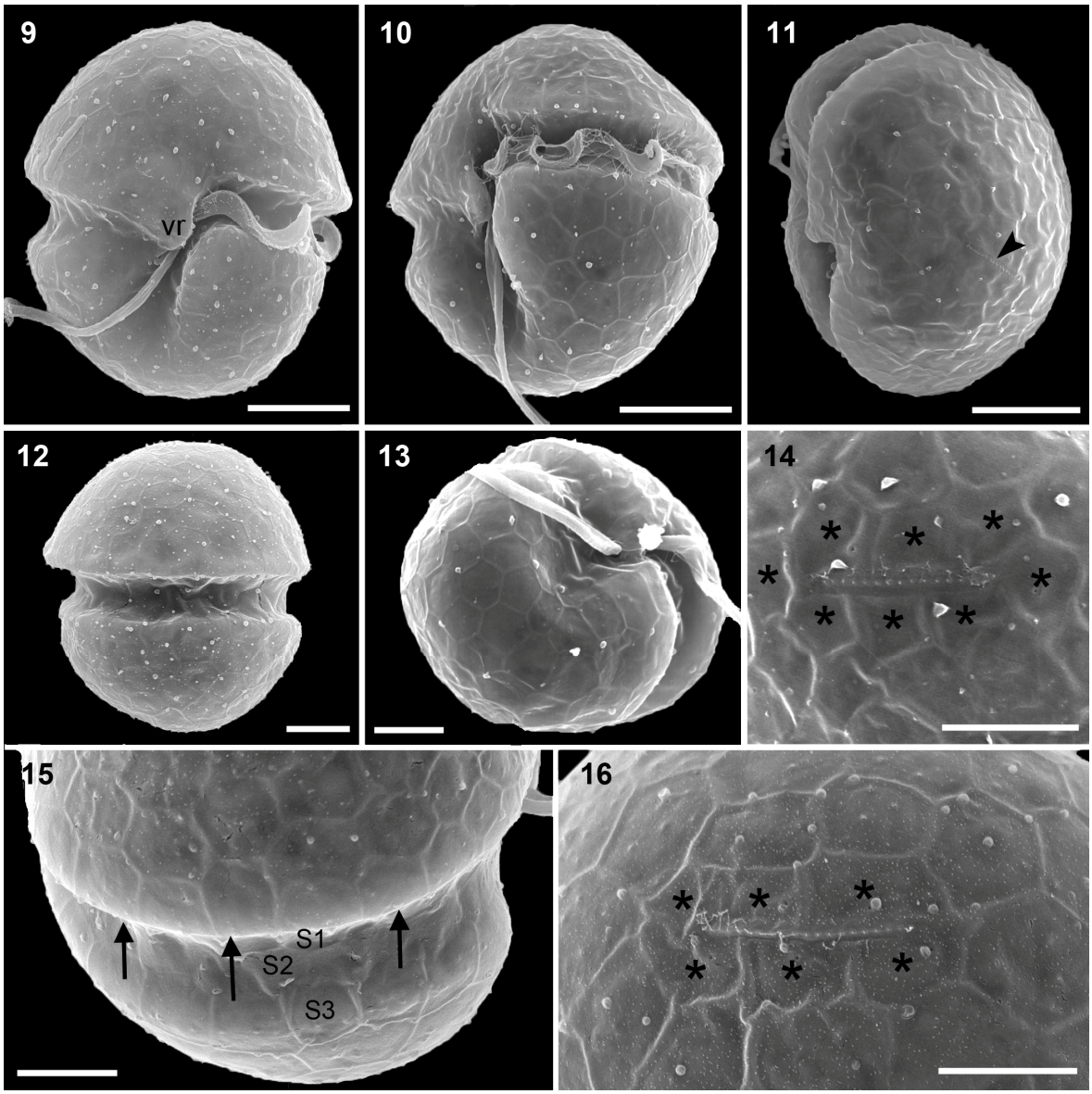
Regarding the division in non-motile stage, two cells almost or already divided were found inside "vesicles" on the bottom of the culture wells (Fig. 5). "Vesicles" with four cells were rarely seen (Fig. 6). Daughter cell release was observed once and occurred through the burst of the "vesicle", releasing two cells that began to swim at that moment. Some of these "vesicles" were followed for several days with the cells inside, behaving like temporary cysts.

Planozygotes, identified by the presence of two longitudinal flagella (Fig. 18), were observed in both culture lines, indicating that *B. andersenii* sp. ined. was able of reproducing sexually and is homothallic. Cysts were also seen in both culture lines. They were smooth, spherical to elongate, usually with almost colorless contents except for one long or several orange to reddish-brown accumulation bodies (Figs 7, 8). As this species also reproduced sexually and the cysts were able to keep for several weeks in the wells, we interpret them as resting cysts or hypnozygotes.

Phylogeny of *B. andersenii* sp. ined.

As the partial sequences of LSU rDNA from MSP2 and MSP8 culture lines were 100% similar, we used only one for the phylogenetic inference based on Bayesian Analysis. The result of this inference is the phylogenetic tree shown in Fig. 21, which shows three well-supported clades (posterior probabilities, pp = 1.0). *Borghiella andersenii*

sp. ined. (boldface in the tree) appears in the clade with other *Borghiella* species, *B. tenuissima* and *B. dodgei.* The species designated as MSP3 is growing in our culture collection and is morphologically identical to *B. andersenii* sp. ined..



Figs 9–16. SEM of vegetative cells of *Borghiella andersenii* sp. ined.. Fig. 9. Ventral view showing the cell shape and plate arrangement. The ventral ridge (vr), the transverse and longitudinal flagella are also visible. Fig. 10. Ventral-side view. Both flagella are present. Fig. 11. Apical view showing the somewhat compressed dorsoventrally cell and the pair of elongate amphiesmal vesicles (PEV, arrowhead). Fig. 12. Dorsal view. Fig. 13. Antapical view. The sulcal area is visible. Figs 14, 16. Detail of the PEV. The asterisks indicates the apical plates surrounding it. Fig. 15. Apical-side view showing the cingular area with three series of plates (S1, S2 and S3). The arrows point to the anterior border of the cingulum. Figs 9–12, scale bar = 5 μm; Figs 13–17, scale bar = 2.5 μm.

DISCUSSION

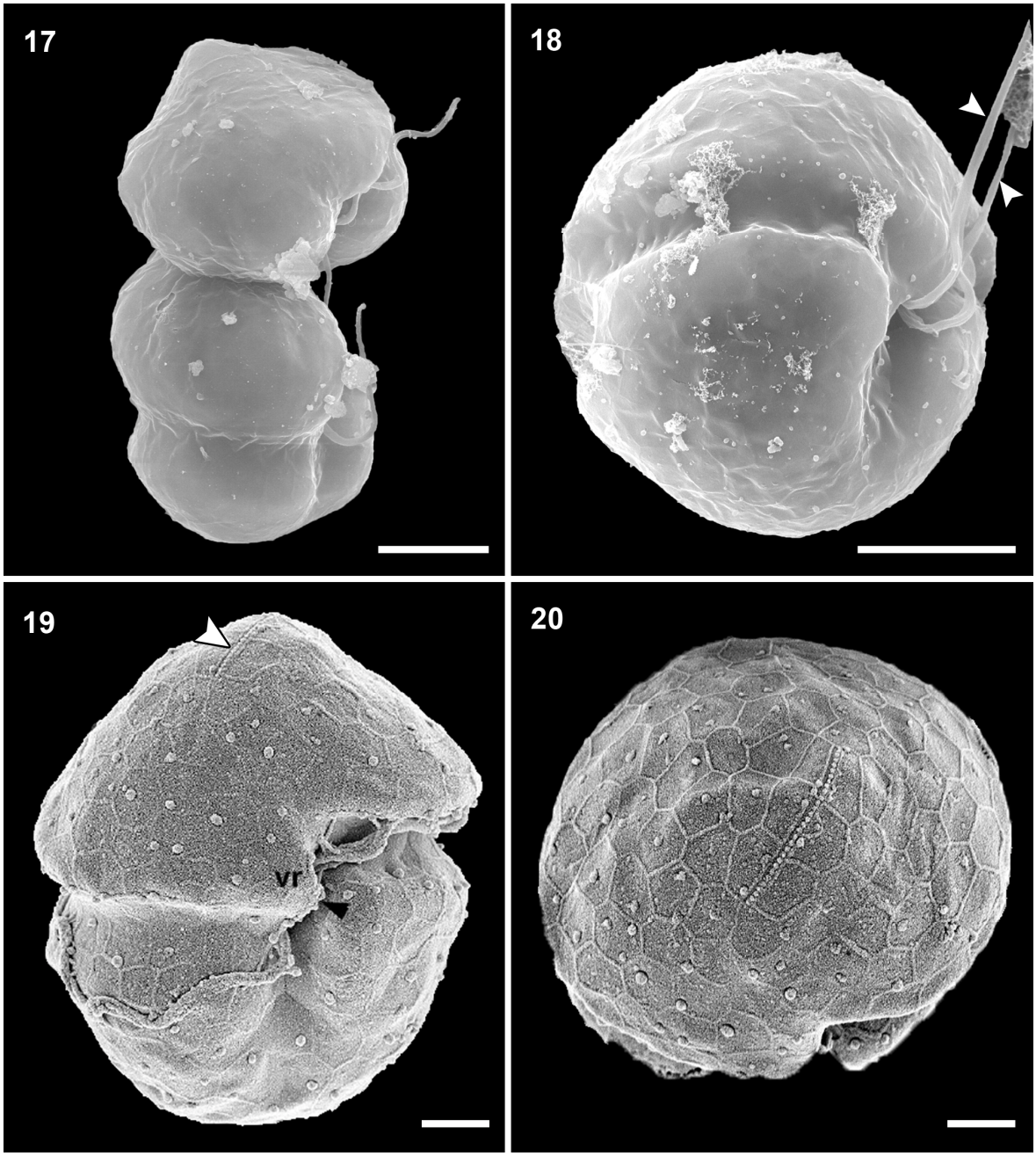
Identity of the organism

The species described in this work presents the main distinctive characters of the genus *Borghiella*, as described by Moestrup *et al.* (2008). It has a cell cover composed of numerous amphiesmal vesicles; a pair of elongate amphiesmal vesicles (PEV) on the epicone, near the apex; golden or yellowish-green chloroplasts; smooth cysts; and is capable of division in the motile stage by fission. The type of eyespot is also an important distinctive character of the genus. In our organism, it was not noticeable through LM observations, a circumstance that was also reported in *B. dodgei* (Moestrup *et al.,* 2008). However, we were able to assert that it also has an eyespot type B, based on ultrastructural observations (Moestrup, personal communication). In the phylogenetic inference based on Bayesian Analysis (Fig. 21) there are three well supported clades, which correspond to the three dinoflagellate families that presently assemble most woloszynkioids, each one having a different type of eyespot. Our species (in boldface) appears, as expected, in the clade corresponding to the family Borghiellaceae, close to the other two *Borghiella* species. Thus, taking into account the morphologic and life cycle aspects referred, and the phylogenetic result, we place this species in the genus *Borghiella*.

Comparison with other *Borgiella* species

Presently, the genus *Borghiella* includes only the two species described at the time of its original description, *B. dodgei* and *B. tenuissima* (Moestrup *et al.,* 2008). The latter is morphologically rather distinct from ours, having a strong dorsoventral flattening that makes it one of the most peculiar woloszynskioid dinoflagellates. The former, on the contrary, is very similar to the present species; the most prominent difference between the two species is related with the cell shape. While *B. dodgei* was described as having an epicone "typically slightly conical-shape" (Moestrup *et al.,* 2008) (Fig. 19), the epicone of *B. andersenii* sp. ined. was usually rounded. At the apical complex level there were also some constant divergences, mainly associated with the size of the PEV, the number of knobs in it and the number of plates surrounding it. The PEV of *B. dodgei* (Fig. 9 in Moestrup *et al.,* 2008; Fig. 20 in this work) is about 6 μm long, has 29 knobs, and, according to the authors, this structure can be lined on each side by three or four plates. In

contrast, the PEV of our species was shorter, usually between 3 and 4 μm, had up to 17 knobs, and was lined on each side by two or three plates.



Figs 17–20. SEM of *Borghiella* species. Fig. 17. Division pair of *B. andersenii* sp. ined. Cells still connected by the epicones. Fig. 18. Planozygote of *B. andersenii* sp. ined.. Two longitudinal flagella marked with arrowheads. Fig. 19, 20. Vegetative cells of *B. dodgei*, adapted from Moestrup *et al.* (2008). Fig. 19. Ventral view showing the cell shape and plate arrangement. The ventral ridge (vr), ventral ridge proper (black arrowhead) and the PEV (white arrowhead) are visible. Both flagella are present. Fig. 20. Apical view showing the PEV. Figs 17, 18, scale bar = 6 μm; Figs 19, 20, scale bar = 2 μm.

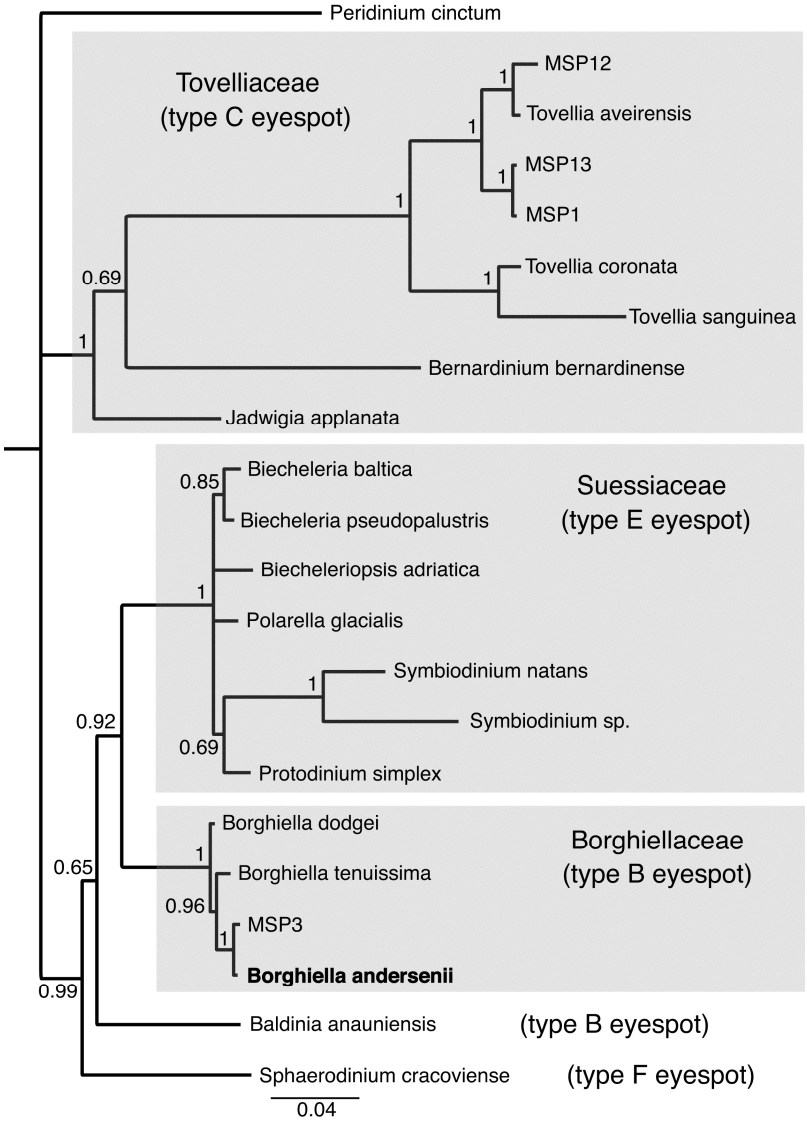


Fig 21. Phylogeny of *Borghiella andersenii* sp. ined. (in boldface) and 21 other dinoflagellates based on partial LSU rDNA sequences, inferred by Bayesian Analysis using MrBayes. Posterior probabilities values are presented at the nodes. *Peridinium cinctum* was used to root the ingroup of the other dinoflagellates. Branch lengths are proportional to the number of base changes. The family clades are in grey boxes with the indication of type of eyespot (*sensu* Moestrup & Daugbjerg, 2007).

Regarding the ways of reproduction, in *B. dodgei* asexual reproduction was only reported in the motile stage, by fission (Moestrup *et al.,* 2008), which, as seen before, is the only way of vegetative reproduction known in Borghiellaceae previously. In *B. andersenii* sp. ined., however, was also observed asexual reproduction in the non-motile stage, inside "vesicles" that resemble the division cysts or zoosporangia described by Stosch (1973) for *Woloszynskia apiculata* Stosch (presently *Tovellia apiculata* (Stosch) Moestrup, K. Lindberg & Daugbjerg). This type of division cysts were also reported in other species now included in the genus *Tovellia* Moestrup, K. Lindberg & Daugbjerg (e.g. Wołoszyńska, 1917; Christen, 1958; Shyam & Sarma, 1975; Pandeirada *et al.,* (submitted; Chapter 3)). In most cases, the number of cells released from a division cyst of a particular species varies from two to eight. In our species, however, the number was less variable:

generally two and, very rarely, four cells were seen. This limited variability was also verified in *Tovellia aveirensis* sp. ined. (Pandeirada *et al.,* (submitted); Chapter 3), although in this case the number of cells released was usually four. The release of cells from the division cyst was observed on several occasions in *T. aveirensis* sp. ined., and usually occurred shortly after the detection of the cysts. In contrast, in *Borghiella andersenii* sp. ined., the release of the cells was seen only once in spite of numerous attempts to observe it, as the division cysts remained for several weeks with the cells inside, suggesting that they might behave as temporary cysts of surprisingly long duration. Sexual reproduction in this species was strongly suggested by the appearance of planozygotes in both culture lines, indicating that it is homothallic (capable of self-fertilization). As seen before, references to sexual reproduction on Borghiellaceae have been based on the observation of apparent resting cysts. In the present species we interpreted cells as those shown in Figs 7, 8 as resting cysts that seemed to remain viable in the wells for several weeks. Within the genus *Borghiella* cysts were only described for *B. tenuissima*, as being smooth, ovoid or globular (Stosch, 1973; Moestrup *et al.,* 2008). This description is reminiscent of the cysts found in the new species, which also had smooth walls, and were spherical or elongate. Nevertheless, as already referred, *B. tenuissima* vegetative cells are morphologically rather distinct from *B. andersenii* sp. ined..

The species described in this work is morphologically very similar to *B. dodgei*, with the main differences at the level of the cell shape and the apical complex, and seems to diverge from it and other members of Borghiellaceae mainly by having a second mode of asexual reproduction. Despite these differences, we initially considered that would be imprudent to establish a new species just based on them, since they might only reflect divergences among populations of the same species, and identified it as *Borghiella dodgei* in a checklist of freshwater dinoflagellates of Portugal (Pandeirada *et al.,* 2013; Chapter 2). However, in the phylogenetic analysis performed later (Fig. 21), B. *andersenii* sp. ined. came out as a sister group to the peculiar *B. tenuissima,* and therefore evolutionarily more separated from *B. dodgei*. This result agreed with that obtained by the other coauthors of this future submission, which have performed a more complete phylogenetic analysis, also based on partial sequences of LSU rDNA and including a higher number of dinoflagellate groups (Daugbjerg, personal communication).

Therefore, taking into account the phylogenetic results and all the differences mentioned above in relation to *B. dodgei*, we consider the organism described in this work as a new *Borghiella* species and name it *Borghiella andersenii* sp. ined.. This is also the first report of asexual reproduction involving division cysts in the Borghiellaceae.

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CHAPTER 5

FURTHER OBSERVATIONS ON WOLOSZYNSKIOIDS

FURTHER OBSERVATIONS ON WOLOSZYNSKIOIDS

Chapters 3 and 4 of the present thesis report on, respectively, the new species *T. aveirensis* sp. ined. and *B. andersenii* sp. ined.. Two other woloszynskioid culture lines, named MSP1 and MSP12, are currently maintained in our culture collection under the following conditions: 18ºC with 12:12 light:dark photoperiod in 4× concentrated L16 medium (Lindström 1991) supplemented with vitamins according to Popovský & Pfiester (1990). MSP1 was established from the isolation of a single cell from a sample collected in a farm pond at Gafanha da Boavista, near Vista Alegre, Ílhavo, Aveiro (40°35'44.70"N, 8°41'49.66"W), on 28 October 2010. MSP12 started from a cell isolated from the same location where *B. andersenii* sp. ined. was found, in a flooded area in Ribeiro da Palha stream, Nariz, Aveiro (40°33'14.42"N, 8°34'5.73"W), on 20 June 2011. In this chapter both species are briefly described under the designations of MSP1 and MSP12. A more complete account of these organisms will require further examination and will be given elsewhere.

MATERIAL AND METHODS

Light microscopy (LM)

Light micrographs of motile cells and cysts were taken with a Zeiss Axioplan 2 imaging light microscope (Carl Zeiss, Oberkochen, Germany) equipped with a DP70 Olympus camera (Olympus Corp., Tokyo, Japan).

Scanning electron microscopy (SEM)

The procedure used to prepare swimming cells of MSP1 was the same as outlined for *Borghiella andersenii* sp. ined. (Chapter 4). Swimming cells of MSP12 were prepared according to the following procedure: 1 ml of culture was fixed for 6 min with 0,1 ml of 4% osmium tetroxide. After fixation the procedure was similar for both species. Fixed cells were retained on Isopore polycarbonate filters with 8 µm pore size (Millipore Corp., Billerica, USA) and washed with milli-Q water for 70 min. The filters with the cells were dehydrated with a graded ethanol series and critical point dried. The filters were glued onto stubs, sputter-coated with gold-palladium and examined with a Hitachi S-4100 (Hitachi High-Technologies Corp., Tokyo, Japan) scanning electron microscope (SEM).

Single cell PCR amplification of LSU rDNA

MSP1 and MSP12 cells were used for PCR amplification. In both cases, swimming single cells from cultures were isolated with a micropipette under the inverted microscope, and transferred to one 0.2-ml PCR tube with 8 µl of milli-Q water, which were frozen at -8ºC for 4 days, before PCR reactions. Cell DNA constituted the template to amplify about 1500 base pairs (bp) of the LSU rRNA gene using the terminal primers D1R (Scholin et al. 1994) and 28-1483R (Daugbjerg et al. 2000). These were added to the PCR tubes with the isolated cells, followed by illustra^{TM} puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK Ltd., Buckinghamshire, UK) containing all other chemicals necessary to the PCR amplification. The reaction occurred in the Biometra–Tprofessional thermocycler (Biometra GmbH, Goettingen, Germany). The thermal profile for MSP1 included one initial cycle of denaturation at 94ºC for 7 min; 35 cycles of each of the following steps: denaturation at 95ºC for 1 min, annealing at 53ºC for 1 min, and extension at 72ºC for 2 min; and the final cycle of extension at 72ºC for 7 min. Thermal profile for MSP12 was the same as described for *B. andersenii* sp. ined. cells of MSP2 culture line, in Chapter 4. The PCR product was loaded on a 1% agarose gel, run for 20 min at 90 V and viewed under a UV light table (Molecular imager chemiDoc XRS System, Bio-Rad Laboratories, Inc., Hercules, California, USA). As the PCR bands of both species were weak, another PCR amplification was performed using the PCR product of the first attempt (nested-PCR). The procedure followed was the same as described for *B. andersenii* sp. ined. (MSP2 culture line) in Chapter 4. PCR and nested-PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's recommendations, and sent to Macrogen Europe (Amsterdam, The Netherlands) for sequence determination in both directions. The sequencing primers used were D1R, D2C, D3A, D3B and 28-1483 (Hansen et al. 2000).

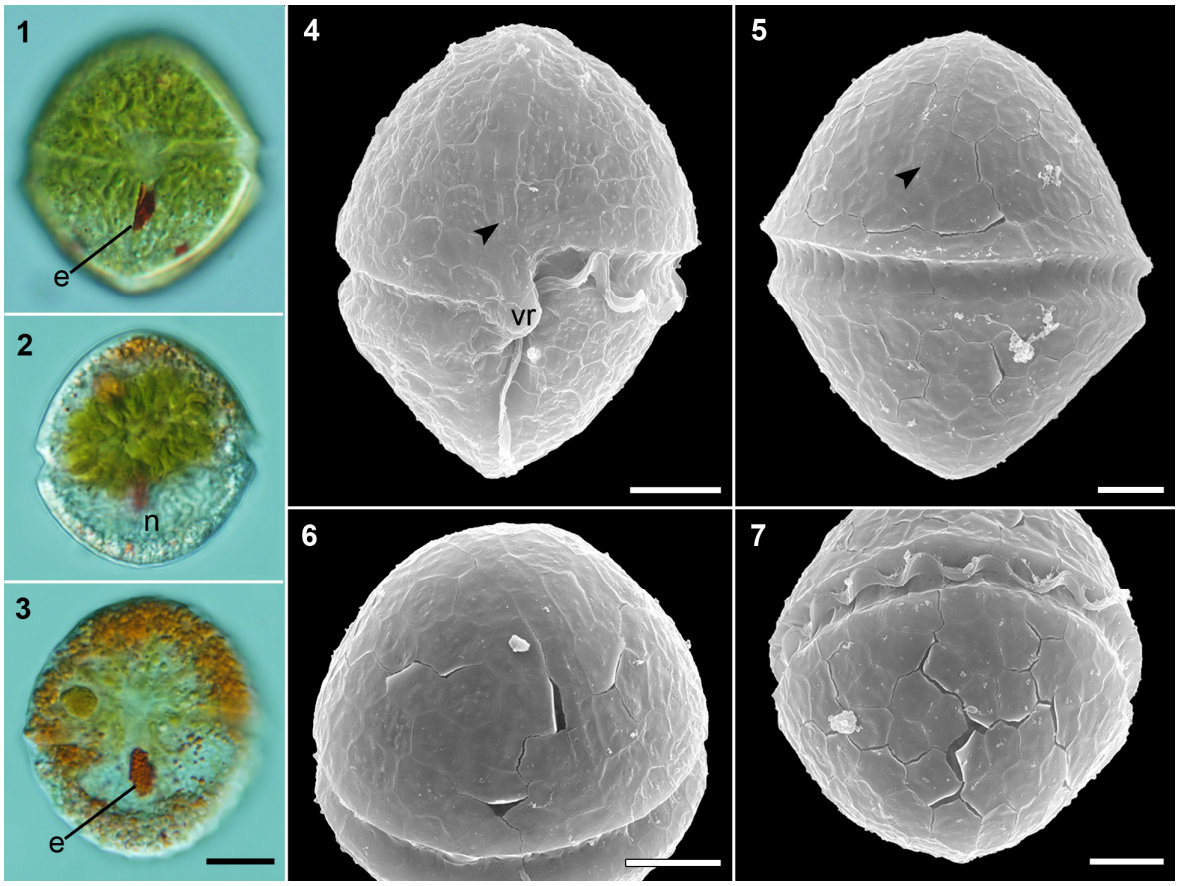
Alignment and phylogenetic analyses

Methods used to align partial LSU rDNA sequences of MSP1 and MSP12 with sequences of other dinoflagellates (mainly woloszynskioids), and produce a phylogenetic tree inferred from Bayesien Analysis were outlined in Chapter 4.

RESULTS

Description of MSP1

Motile cells are illustrated in Figs 1–7. The epicone of vegetative cells was ovate-conical and the hypocone usually plainly conical or even pointed (Figs 1, 4, 5). The cingulum was displaced about one cingulum width and divided the cell into approximately equal-sized parts (Figs 1–5). Cells were not compressed dorsoventrally at the cingulum level, or only slightly so, and the hypocone was obliquely flattened (not shown). Cells were 22–36.5 μm long and 19–33 μm wide (n = 30). A bright-red extraplastidial eyespot was located along the sulcal area (Figs 1, 3). Chloroplast lobes were yellowish-green and seemed to radiate from the cell centre toward the periphery (Figs 1, 2, 3). The nucleus was located in the hypocone (Fig. 2). The cell cover was mainly formed by pentagonal or hexagonal amphiesmal vesicles roughly arranged in latitudinal series, usually with six or seven series on the epicone and four or five on the hypocone (Figs 4–7). The cingulum was composed of two series of vesicles, the anterior one made of vesicles abutting the sharply defined anterior edge, whereas the roughly hexagonal vesicles of the posterior row extended into the hypocone over the rounded posterior cingulum edge (Fig. 5). A row of feebly marked knobs is visible on Fig. 5 along the posterior edge of the cingulum. A line of narrow vesicles (ALP) started on the ventral side, near the proximal end of the cingulum, and extended over the apex of the cell ending on the dorsal side, two or three rows of plates from the anterior border of the cingulum (Figs 4–6). A somewhat projecting ventral ridge was observed in the upper part of the sulcus (Fig. 4). Cells changed from yellowish-green to reddish-brown as the culture became older, or few days after their transference to medium without nitrogen. The reddish-brown cells were usually larger and more round, and swam more slowly. Cells that were strongly flattened dorsoventrally (Fig. 3) were occasionally observed, both in batches with yellowish-green and with reddish-brown cells. Asexual reproduction involved the formation of division cysts, from which were usually released four cells (not shown). Planozygotes or cysts were never observed.



Figs 1–7. Vegetative cells of MSP1 species. Figs 1–3. LM of vegetative cells. Fig. 1. Ventral view showing the eyespot (e). Fig. 2. Dorsal view showing the nucleus (n) in the hypocone, some accumulation of brown/red bodies at the periphery of the epicone and the apparent radiating arrangement of the chloroplasts. Fig. 3. Ventral view of a round brown/reddish cell. It is visible a great accumulation of brown/red bodies mostly at the cell periphery. Figs 4–7. SEM of vegetative cells. Fig. 4. Ventral view showing the cell shape and plate arrangement. The ventral ridge (vr) and both flagella are visible. The arrowhead points to the ventral end of the line of narrow vesicles (ALP). Fig. 5. Dorsal view. ALP dorsal end indicated by an arrowhead. Fig. 6. Apical/Dorsal view showing the ALP. Fig. 7. Antapical view. Figs 1-3 at the same scale, scale bar = 10 µm. Figs 4-7, all scale bars = 5 µm.

Description of MSP12

Motile cells of MSP12 are illustrated in Figs 8, 9, 11–16. The epi- and hypocone of vegetative cells were usually ovate-conical (Figs 8, 9, 11–13). The hypocone was sometimes distinctly apiculated, mainly in lateral view (Fig. 15). Epi- and hypocone were similar in size, or the former was somewhat larger (Figs 8, 9, 11, 12, 15). The cingulum was displaced about one cingulum width (Figs 8, 11). Cells were not compressed dorsoventrally at the cingulum level, or only slightly so, and the hypocone was obliquely

flattened (not shown). Cells were 16–31 µm long and 11–26 µm wide (n = 30). A bright-red extraplastidial eyespot was located in the upper part of the sulcus (Fig. 8). Chloroplast lobes were yellowish-green and appeared radiating from the cell centre toward the periphery (Figs 8, 9). The nucleus was located in the hypocone (Fig. 9). The cell cover was mainly formed by pentagonal or hexagonal amphiesmal vesicles roughly arranged in 5-7 latitudinal series on the epicone and 3–4 on the hypocone (Figs 11–13, 15). The cingulum contained two series of vesicles, the anterior one abutting the straight anterior edge, whereas the roughly hexagonal vesicles of the posterior row extended into the hypocone over the rounded posterior cingulum edge (Figs 12, 15). In some cells a row of knobs was barely visible along the posterior edge of the cingulum (not shown). An apical line of narrow vesicles (ALP) started near the proximal end of the cingulum, on the ventral side, and extended over the apex of the cell, two or three rows of plates from the anterior border of the cingulum (Figs 12, 14, 15). A ventral ridge was observed at the cingular-sulcal junction (Fig. 11). Cells with very different sizes were frequently observed in the same batch (Fig. 16, white and black arrowheads point to small and very large cells, respectively). Asexual reproduction involved the formation of division cysts, from which were usually released four cells (not shown). Planozygotes were seen, identified by the presence of two longitudinal flagella (not shown). Apparent resting cysts were nearly elipsoid, with signs of paracingulum and a smooth wall with some spiny processes (Fig. 10).

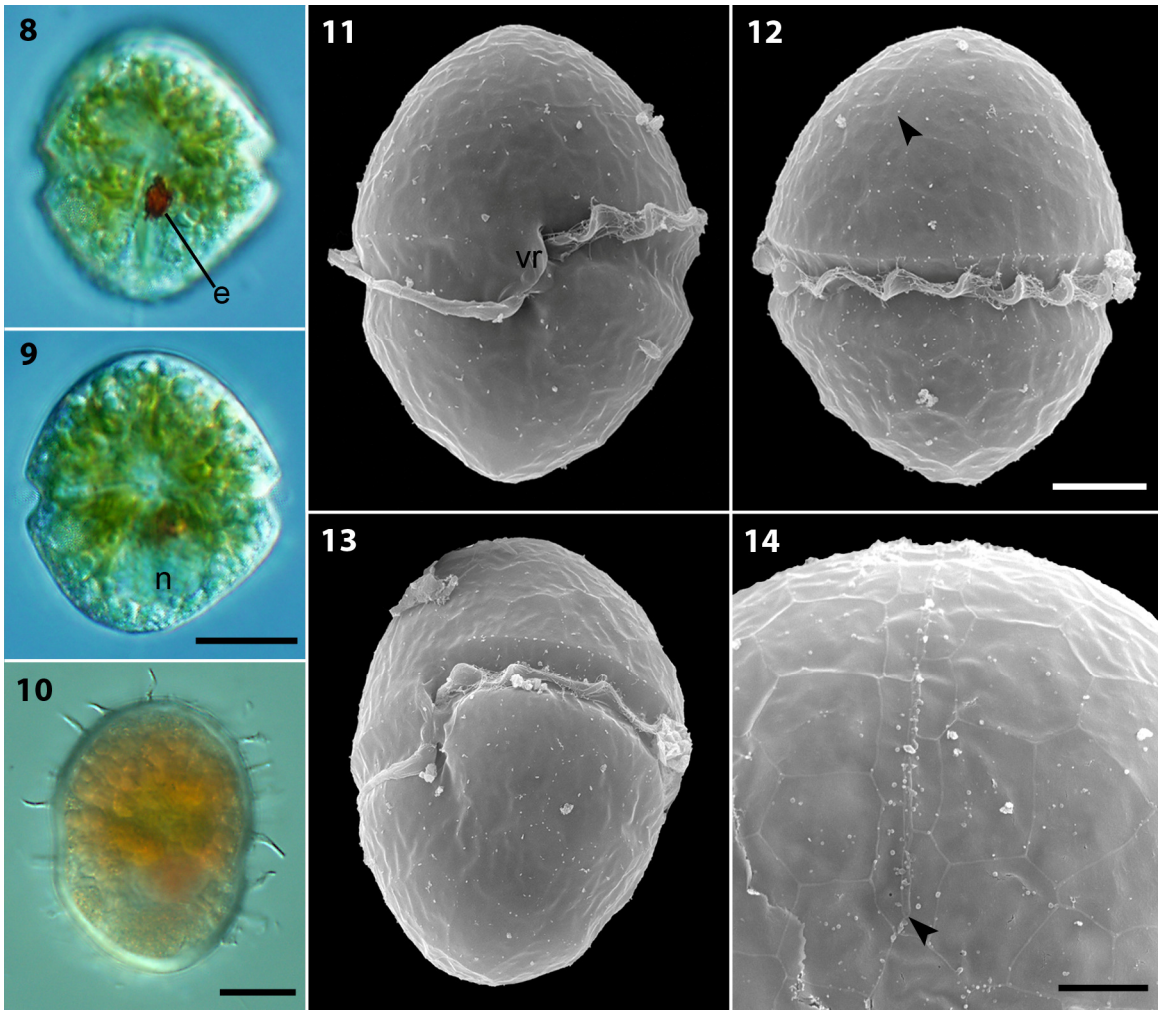
Phylogeny of MSP1 and MP12

A phylogenetic tree including these two strains, inferred from Bayesien Analysis, is shown in Chapter 4, Fig. 21 (p. 116). The tree displays three well-supported clades (posterior probabilities, pp = 1.0). MSP1 and MSP12 appear in a clade corresponding to the family Tovelliaceae, and form a clade together with *T. aveirensis* sp. ined..

DISCUSSION

Identity of the organisms

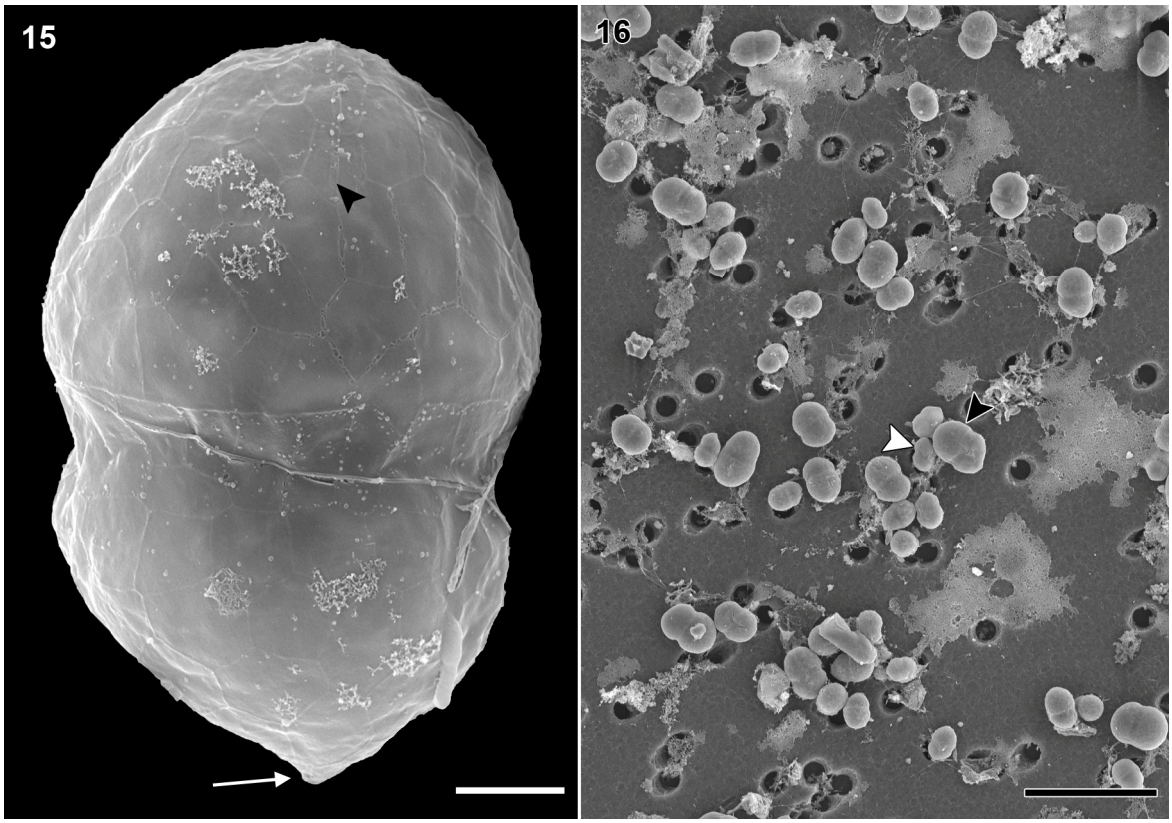
Both strains showed the morphological characters typical of the genus *Tovellia*, *viz.* the distinctive line of narrow plates on the epicone (ALP *sensu* Lindberg et al. 2005) and the extraplastidial eyespot type C *sensu* Moestrup & Daugbjerg (2007). Furthermore, in the



Figs 8–14. MSP12 species. Figs 8, 9. LM of vegetative cells. Fig. 8. Ventral view showing the eyespot (e). Fig. 9. A deeper focus of cell in Fig. 8 showing the nucleus (n) in the hypocone and the apparent radiating arrangement of the chloroplasts. Fig. 10. LM of cyst with few simple spines. Figs 11–14. SEM of vegetative cells. Fig. 11. Ventral view showing the cell shape and plate arrangement. Ventral ridge (vr) and both flagella visible. Fig. 12. Dorsal view. ALP is visible with its dorsal end indicated by an arrowhead. Fig. 13. Antapical view. Fig. 14. Detail of the ALP. The arrowhead point to its ventral end. The following Figs are at the same scale: Figs 8, 9; Figs 11-13. Figs 8-10, all scale bars = 10 µm; Figs 11-14, all scale bars = 5 µm.

phylogenetic tree (Chapter 4, Fig. 21 on p. 116), they both grouped with other species of *Tovellia*, namely *T. coronata, T. sanguinea* and *T. aveirensis* sp. ined.. In particular, they were closer to the latter, with the MSP12 coming out as a sister taxon to *T. aveirensis* sp. ined., and the MSP1 as a sister group to these two. The evolutionary affinity among the three species is also suggested morphologically, since they share a set of features that are perhaps significant in the context of the genus, such as a row of knobs at the posterior edge of the cingulum, surrounding the cell; the absence of a hexagonal plate on the hypocone

around which latitudinal series of vesicles are arranged; and chloroplasts which appeared radiating from a central pyrenoid complex. The affinity with *Tovellia* is therefore reliably demonstrated.



Figs 15, 16. SEM of MSP12 species. Fig. 15. Dorso-lateral view showing the ALP (arrowhead points to the dorsal end) and the hypocone ending in an apiculus (arrow). Fig. 16. Several cells of MSP12 in lower magnification. Notice a marked difference in cell size. The white arrowhead points to a smaller cell, and the black one to a large cell. Fig. 15, scale bar = 2.5 μm; Fig. 16, scale bar = 60 μm.

Comparison with other *Tovellia* species

MSP1

MSP1 showed a peculiar ability to change colour from greenish to reddish-brown. Sometimes this was accompanied by a change in shape, size and motility. Cells became larger, more round and dorsoventrally flattened; their movement became also slower. These modifications occurred usually as cultures aged or a few days after being transferred to nitrogen-defficient medium. The accumulation of brown or red bodies in the cytoplasm is perhaps a response to stress conditions, such as nutrient depletion. Within the genus

Tovellia yellowish-green, brown and red species have all been reported, which seem to maintain their colour during all life cycle (Stosch 1973; Lindberg et al. 2005; Moestrup et al. 2006; Chapter 3). Colour change were reported for planozygotes, which became darker as a result of progressive accumulation of reserves, such as starch (Stosch 1973). Indeed, some of the reddish-brown MSP1 cells were reminiscent of planozygotes in the process of encysting, because they were large and swam slowly, stopping several times on the bottom of the culture wells, sometimes for long periods. However, after some time lying on the bottom of the wells, cleavage furrows appeared in their cytoplasm, indicating that they were dividing. Division of reddish-brown and yellowish-green cells have been recorded and, in both cases, involved the formation of division cysts, from which were released four cells, as seen in *T. aveirensis* sp. ined. (Chapter 3). Besides the ability of changing its colour, MSP1 has a distinct shape, with the epicone ovate-conical and the hypocone usually conical or pointed, recalling the brown *T. apiculata* (previously *Woloszynskia apiculata*), which also possesses radiating chloroplasts and lacks the hexagonal plate on the hypocone around which the latitudinal series are arranged. This is normally replaced by an apiculus composed of 3–4 platelets (Stosch 1973). Nevertheless, to our knowledge, there are no references to colour changes in *T. apiculata* and it has more latitudinal series than MSP1 (Stosch 1973). Another particularity of MSP1 is that it has never produced cysts, even when inoculated in media without nitrogen to attempt triggering the sexual process. This might indicate that it is not capable of self-fertilization (and is therefore heterothallic), or that it does not reproduce sexually at all. The significance of the strongly flattened cells that were occasionally observed in MSP1 batches is, for the time being, unknown.

Therefore, it is possible that MSP1 represents a previously undescribed species of *Tovellia* that has the unusual ability of changing its colour from greenish to reddish-brown, and which, as suggested in the phylogenetic tree of Chapter 4 (Fig. 21), may occupy a 'transitional' position between the yellowish-green and the reddish species *T. coronata* and *T. sanguinea*. However, hypotheses concerning the phylogenetic relationship of *Tovellia* species are rendered largely speculative by the limited number of species for which the LSU rDNA sequence is available.

Further research planned for this species will include verifying whether it is a new *Tovellia* species by: a thorough search of the literature for reports of colour modifications

in other potential members of this genus; confirming the type of eyespot and chloroplast arrangement by transmission electron microscopy; experimenting with the cultures to determine the conditions that determine the change in colour; identifying the red compound; exploring the variations of genetic activity and expression between yellowish-green and red cells using metabolomic or proteomic approaches; testing the hability of the cells to reproduce sexually by crosses with other strains, in particular with MSP13 (included in the phylogenetic tree of Chapter 4, Fig. 21, p. 116), which were isolated from the same location and also display changes of colour. These experiments will be conducted in culture wells with normal medium or deficient in nutrients, such as nitrogen or phosphorus, or both. The strains also need to be followed through their life cycle to try to find out the origin and significance of the strongly flattened cells.

MSP12

MSP12 has a cell shape very similar to MSP1, with an epicone ovate-conical and a hypocone that varied from conical to strongly apiculated in some cells. Nevertheless, MSP12 cells were always yellowish-green. Because of this, one species that could be easily associated with MSP12 would be *T. apiculata*, which, as seen before, also has radiating chloroplasts (Stosch 1973). Nevertheless, besides the apiculus in the hypocone, the epicone of *T. apiculata* can also be projected along the ALP (Stosch 1973), and this was never seen in our species. In addition, MSP12 has fewer series of plates and produces different cysts. These are similar to the cysts of *T. aveirensis* sp. ined., usually nearly spherical or elongate, with signs of paracingulum and a smooth or spiny wall. *Tovellia apiculata* cysts, in contrast, show a morphology more common in *Tovellia*, with a paracingulum, two axial horns and lateral protuberances or spines (Stosch 1973). As already referred, MSP12 grouped as a sister taxon with *T. aveirensis* sp. ined. and would therefore be expected to have a similar cyst. Morphologically, these two species are mainly distinguishable by cell shape, with *T. aveirensis* sp. ined. being usually ovoid or nearly spherical. In plate arrangement and in the position of eyespot there are also some differences: MSP12 cells had 3 or 4 series of amphiesmal vesicles on the hypocone, and *T. aveirensis* sp. ined. usually 4; the eyespot of MSP12 tended to be near the upper part of the sulcus, while in *T. aveirensis* sp. ined. it occupied most of the sulcal area. Cells extremely different in size were frequently observed in cultures of MSP12. Some of them were tiny

(about 16 μm long) and others large (over 30 μm long); these might correspond to gametes and planozygotes, respectively. Planozygotes were recorded and had lengths similar to those of the large cells (27–31 μm, n = 4). The presence of planozygotes in the cultures suggests that this species is capable of self-fertilization, which would constitute the first report of homothally in a species of *Tovellia*. However, planozygotes and cysts have been seen few times, further studies will be necessary to confirm this hypothesis. An interesting experiment to carry out would be to inoculate cells of MSP12 and *T. aveirensis* sp. ined. in the same culture wells to verify if sexual reproduction would be possible between the two species. This could be, for example, indicated by the production of cysts, since when two compatible lines of *T. aveirensis* sp. ined. were joined, cysts were massively produced. If sexual reproduction was not verified, this would argue for considering them as different biological species, although the possibility of incompatible strains of the same species must also be considered.

Thus, taking everything into account, the species previously described is a potential new *Tovellia* species, which may represent a sister taxon to *T. aveirensis* sp. ined.. Future goals for this species will be: verifying if it may really be considered a new species of *Tovellia*, by comparing with published reports of *Tovellia*-like organisms; performing the experiments referred above with *T. aveirensis* sp. ined. cells in order to verify if they are able to reproduce sexually; identifying the type of eyespot and chloroplasts arrangement by transmission electron microscopy; and studying its life cycle, which will aid to our understanding of the high divergence in the cell size and help determine if MSP12 is homothallic.

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CHAPTER 6

CLOSING REMARKS

CLOSING REMARKS

The dinoflagellates are a diverse group of protists with unusual intra- and extracellular features. The traditional classification of the group, based mainly on external morphology, is being replaced by a classification supported by both molecular and ultrastructural data, such as the type of eyespot, the organization of the apical complex and the features of the resting cyst (Chapter 1; Moestrup & Daugbjerg 2007). As a result, it has been shown that several taxa established on the basis of particular morphological characters are no longer supported and that the taxonomic position of several species needs to be re-evaluated. The genus *Woloszynskia*, created to include *Gymnodinium*-like species covered with numerous thin plates, is one example. Recent revisions on the basis of ultrastructure and LSU rDNA phylogenies confirmed *Woloszynskia* as a polyphyletic genus comprising several taxonomic groups, and new genera and families have been created to hold its members (Chapter 1; Moestrup et al. 2009a, b).

Taxonomic knowledge on the diversity and geographic distribution of freshwater dinoflagellates in continental Portugal was compiled in a checklist for the first time (Pandeirada et al. 2013) and presented here (Chapter 2). The list was built from 37 references, to which personal observations were added with data for 12 previously unreported taxa. Reported names were assembled into 51 entries, of which one was considered a *nomen dubium*, corresponding to 49 species and one form included in 24 genera of dinoflagellates.

Most records of freshwater dinoflagellates for Portugal resulted from studies conducted with the main purpose to collect information about the general composition of phytoplankton communities in lagoons and reservoirs (e.g. Nauwerck 1959; Oliveira 1982). Only recently have studies concentrated on ultrastructural and phylogenetic aspects of selected species (e.g. Craveiro et al. 2009; Calado et al. 2009). A total of 39 species is cited in previously published works, with *Gymnodinium* and *Peridinium* constituting the genera with the highest number of cited species. Woloszynskioids or related species are found in several different genera: *Gymnodinium excavatum* (Oliveira 1982), *Gymnodinium neglectum* (Nauwerck 1959), *Gymnodinium ordinatum* (Nauwerck 1959), *Gyrodinium pascheri* (Nauwerck 1959, 1962), *Prosoaulax lacustris* (Calado & Moestrup 2005), *Prosoaulax multiplex* (Calado & Moestrup 2005), *Esoptrodinium gemma* (Calado et al. 2006) and *Opisthoaulax vorticella* (Calado 2011). Taking into account the recent

taxonomic revisions, some of the above-mentioned *Gymnodinium* and *Gyrodinium* species were included in the checklist as species of *Biecheleria* and *Jadwigia*, whereas others were provisionally maintained in *Woloszynskia* (i.e., *W. ordinata* and *W. pascheri*).

Added to the list was another woloszynskioid, *Borghiella dodgei*, and the Tovelliaceae *Opisthoaulax fastigata*, two new species for Portugal (Calado 2011). The former was identified as *B. dodgei* taking into account its high morphological similarity with the cells shown in Moestrup et al. (2008), although the more rounded epicone and the shorter apical complex, with fewer knobs and lined on each side by two to three apical plates, was noted as divergent in relation to typical *B. dodgei*. However, closer examination of our cultures of this *Borghiella* species, including LSU rDNA sequencing, revealed the closer relatedness of the Portuguese specimens to *B. tenuissima* than to *B. dodgei* (Chapter 4). Morphological and genetic comparisons between our material and a strain isolated from Scotland that is currently under study in the University of Copenhagen revealed that they were identical and represented a new species. A new *Borghiella* species, *B. andersenii* sp. ined., found in freshwater from Portugal and Scotland, and named in honor to Prof. Robert A. Andersen, was therefore described in Chapter 4. *Borghiella andersenii* was able to reproduce asexually both in the motile stage, by fission, and in the non-motile stage, with production of division cysts. Division in the non-motile stage had not been previously reported within the family Borghiellaceae. Furthermore, stronger evidences of sexual reproduction than previously available for this family have been also found in *B. andersenii* sp. ined., namely planozygotes and cells that may represent resting cysts (Chapter 4).

Another new woloszynskioid confirmed by a phylogeny based on partial LSU rDNA sequences was presented in this work (Chapter 3). It belongs in the family Tovelliaceae, genus *Tovellia*, and was named *T. aveirensis* sp. ined., since it has been collected in a freshwater tank at the University of Aveiro Campus, Aveiro, Portugal. The most peculiar feature of this species is related with its life cycle and consists in the production of a resting cyst with paracingulum and numerous branched spines; these cysts differ not only from the bipolar and less ornamented *Tovellia* cyst, but also from all other cysts described from woloszynskioid dinoflagellates (described in Moestrup et al. 2009a; Chapters 1, 3). The motile cells differed morphologically from other *Tovellia* species mainly by having a row of knobs placed along the posterior edge of the cingulum, surrounding the cell, and by

lacking a hexagonal-octogonal, prominent antapical plate surrounded by series of plates on the hypocone (Chapter 3).

Two other wolosynskioids, labelled as MSP1 and MSP12, were briefly described and discussed in Chapter 5. Both morphological and phylogenetic results suggested that they are two new *Tovellia* species, evolutionarily close to *T. aveirensis* sp. ined.. They have a very similar shape, with an ovate-conical epicone and a conical hypocone, which can be pointed in MSP1 and apiculated in MSP12. Nevertheless, MSP1 has the unusual ability of changing its color from greenish to reddish-brown, whereas MSP12 has so far remained yellowish-green throughout different treatments in culture. Such colour change within *Tovellia* is still insufficiently understood; in our cultures it apparently occurred as a response to stress conditions, such as nutrient depletion.

A relevant number of freshwater dinoflagellates is currently known from Portugal, which is manifest in the 48 species (excluding the incorrectly identified *B. dodgei*) and one form presented in the checklist of freshwater dinoflagellates for the country. To these must be added the recently described *Theleodinium calcisporum* Craveiro, Pandeirada, Daugbjerg, Moestrup & Calado, a peridinioid that represents the first freshwater species to be found producing a calcified cyst (Craveiro et al. 2013). The new *T. aveirensis* sp. ined. (already submitted) and *B. andersenii* sp. ined. (in preparation), and perhaps MSP1 and MSP12, if they prove to be distinct from currently recognized species, must be added to the list. Woloszynskioids are, as documented in this thesis, difficult dinoflagellates to identify, and even with light and scanning electron microscopy distinction of different species is a delicate process, as exemplified for *B. andersenii* sp. ined. This draws attention to the importance of using molecular techniques in dinoflagellate identification, together with ultrastructural information, to supplement or in some cases replace the traditional criteria of classification.

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